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ANALYTICAL METHODS FOR THE QUANTIFICATION OF PERSISTENT ORGANIC POLLUTANTS IN SERUM AND NICOTINE ENANTIOMERS IN ELECTRONIC CIGARETTES

by
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ABSTRACT
OF THE THESIS OF

Cynthia Artin Antossian for Master of Science
Major: Chemistry

Title: Analytical Methods for the Quantification of Persistent Organic Pollutants in Serum and Nicotine Enantiomers in Electronic Cigarettes

Obesity and tobacco use are two major risk factors for non-communicable diseases in Lebanon. In this thesis, studies focused on understanding the role and determining the levels of certain components that contribute to these health risks.

One of the factors leading to obesity is the exposure to persistent organic pollutants (POPs), especially during fetal development. POPs, such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), are known to be endocrine disrupting chemicals that interfere with metabolism and disrupt normal homeostatic controls over adipogenesis and energy balance. In this study, a gas chromatography method that can be used to measure POPs in serum samples collected from the cord blood and maternal blood is developed. In addition, a systematic review and meta-analysis is performed to analyze the associations between prenatal exposure to POPs and childhood obesity. Results obtained from the systematic review and meta-analysis indicated that prenatal exposure to DDE, but not PCBs, was significantly associated with an increase in BMI z-score and overweight risk in childhood.

Tobacco use, on the other hand, is associated with various health effects. For this reason, the Tobacco Control Act provided the Food and Drug Administration authority to regulate all tobacco products. As a result, manufacturers of electronic cigarettes (ECIGs) started using synthetic nicotine instead of tobacco-derived nicotine, since synthetic nicotine was not mentioned as part of the regulation. Thus, high interest in discerning between tobacco-derived and synthetic nicotine has emerged. Tobacco-derived nicotine is mainly found as (S)-nicotine (>99%), while synthetic nicotine usually contains a racemic mixture 50:50 of (R) and (S) enantiomers. In addition, nicotine enantiomers are known to be pharmacologically and toxicologically different. (R)-nicotine being less toxic than the (S) enantiomer, might be used as therapeutic agent for neurodegenerative diseases and tobacco smoking addiction. In this study, a liquid chromatography method that can be used to quantify (R) and (S) enantiomers of nicotine in e-liquid and aerosol samples from ECIGs is developed. Results obtained from the analysis of nicotine enantiomers indicated that (R)/(S) interconversion does not take place during vaping and the form of nicotine, whether (R) or (S), that enters the body will be the same as that initially present in the e-liquid.

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ABBREVIATIONS

ACh	Acetylcholine
ACN	Acetonitrile
AGP	α-Acid Glycoprotein
AUBMC	American University of Beirut Medical Center
BCF	Bioconcentration Factor
BMI	Body Mass Index
CI	Confidence Interval
CINAHL	Cumulative Index of Nursing and Allied Health Literature
CO	Carbon Monoxide
DAD	Diode Array Detector
DCM	Dicholoromethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
ECIG	Electronic Cigarette
Embase	Excerpta Medica Database
ER	Estrogen Receptor
FDA	Food and Drug Administration
GC	Gas Chromatography
GSH	Glutathione
HCB	Hexachlorobenzene
HPLC	High Performance Liquid Chromatography

ID	Internal Diameter
K_{OW}	Octanol-Water Partition Coefficient
LD_{50}	Median Lethal Dose
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MDA	Malondialdehyde
$MeSO_2$	Methylsulfone
MS	Mass Spectrometer
nAChRs	Nicotinic Acetylcholine Receptors
NCD	Non-Communicable Disease
NMDA	N-methyl-D-aspartate
NO	Nitrogen Monoxide
OCP	Organochlorine Pesticide
OH	Hydroxyl
PCB	Polychlorinated Biphenyl
PCB-20	2,3,3'-Trichlorobiphenyl
PCB-35	3,3',4-Trichlorobiphenyl
PCB-101	2,2',4,5,5'-Pentachlorobiphenyl
PCB-118	2,3',4,4',5-Pentachlorobiphenyl
PCB-138	2,2',3,4,4',5'-Hexachlorobiphenyl
PCB-153	2,2',4,4',5,5'-Hexachlorobiphenyl
PCB-180	2,2',3,4,4',5,5'-Heptachlorobiphenyl
PCB-209	Decachlorobiphenyl

PG	Propylene Glycol
$PM_{2.5}$	Particulate Matter
POP	Persistent Organic Pollutant
PPARγ	Peroxisome Proliferator Activated Receptor Gamma
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RR	Risk Ratio
RSD	Relative Standard Deviation
rpm	rotations per minute
SE	Standard Error
SIM	Single Ion Monitoring
SPE	Solid Phase Extraction
TCMX	Tetrachloro-m-xylene
TTR	Thyroid Transport Protein
VG	Vegetable Glycerin
WHO	World Health Organization

CHAPTER I

PERSISTENT ORGANIC POLLUTANTS AND OBESITY

A. Persistent Organic Pollutants

1. Introduction to POPs

Persistent organic pollutants (POPs) are endocrine disrupting chemicals that are persistent in the environment, highly lipophilic, and bio-accumulate in the human body [1]. Due to these properties and the fact that they may cause adverse health effects on humans including cancer and damage to the hormonal, nervous, reproductive, and immune systems, the production and use of POPs were eliminated or restricted according to the Stockholm Convention on Persistent Organic Pollutants [1]. Initially, the Stockholm Convention, which was signed on 22 May 2001 and effective from 17 May 2004, included a list of twelve POPs [2].

Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) are among the twelve POPs listed under the Stockholm Convention [2].

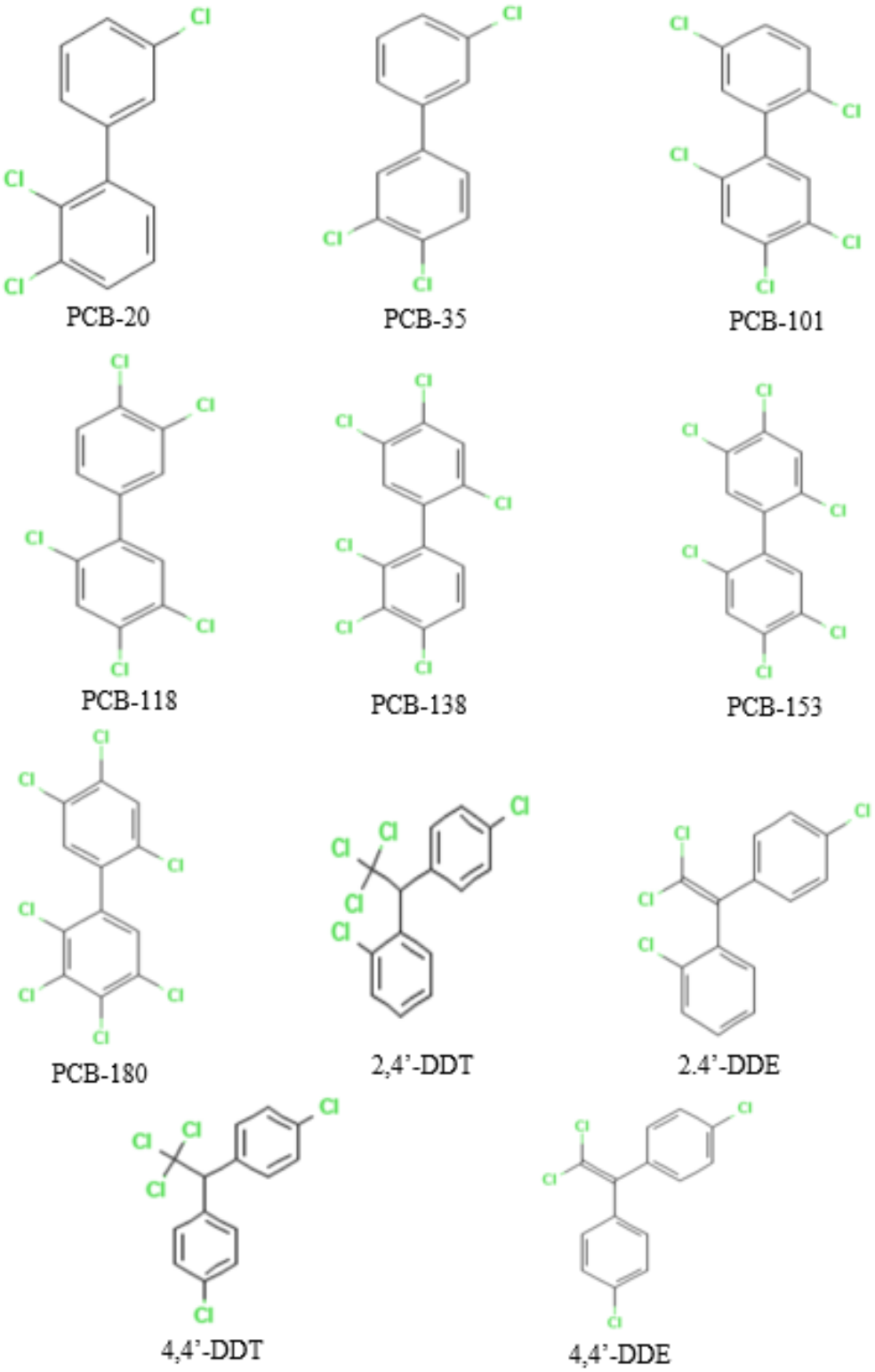

Figure 1: Structure of common PCBs and OCPs

2. Sources

Polychlorinated biphenyls are industrial organochlorine chemicals that are chemically inert, have low vapor pressures, are inexpensive to produce, and are excellent electrical insulators [3]. As a result of these properties, they have many commercial applications. For instance, they can be used as heat exchange fluids in electrical transformers and capacitors, as plasticizers, hydraulic fluids, lubricants, surface coatings, adhesives, and printing inks [4]. In Lebanon, the primary source of PCBs was the insulating oils used by Lebanon's electricity company [5]. These insulating oils contained Aroclor 1260, which is 99% PCBs with five to eight chlorine atoms [5].

Organochlorine pesticides are chemicals that are used to control insects or pests [3]. Among the first known OCPs that was used extensively during World War II is DDT, dichlorodiphenyltrichloroethane [6]. DDT was used as an insecticide, mainly to control mosquitoes involved in the transmission of malaria disease [6]. DDT in the environment is transformed into DDE, dichlorodiphenyldichloroethylene, by DDT-resistant insects that detoxify DDT by this transformation [3]. Other examples of OCPs include aldrin, chlordane, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, and toxaphene.

3. Properties

POPs, such as DDT and PCBs, have high solubility in fat and very low solubility in water. This property of POPs is responsible for their bioaccumulation in living organisms. The two main processes by which POPs bioaccumulate are bioconcentration and biomagnification.

a. Bioconcentration

Bioconcentration is a process by which chemicals bioaccumulate in aquatic organisms through non dietary pathways [7]. The extent to which POPs bioconcentrate in aquatic organisms depends on the bioconcentration factor (BCF), which is defined as the equilibrium ratio of the concentration of the chemical in the aquatic organism relative to its concentration in the surrounding water [3]. Since the main factor that affects bioconcentration of a chemical is its hydrophobicity, BCF can be estimated from the octanol-water partition coefficient K_{ow} by log-linear regression models [8]. This is due to the fact that partitioning of a substance between water and octanol, which is used as a surrogate for the lipid of the organism, defined by K_{ow}, is a measure of hydrophobicity [9]. The higher the K_{ow}, the more likely a substance is to be adsorbed on organic matter in aquatic sediments, which will consequently enter the aquatic organism. Thus, substances with log K_{ow} values between 4 and 7 bioconcentrate the most, since if log k_{ow} is greater than 7, it will not be bioavailable due to strong adsorption to sediments [3]. However, other than the lipid content of the organism, there are other factors that also affect bioconcentration, such as the ability of an organism to metabolize the substance and thus excrete it, preventing accumulation [7].

b. Biomagnification

Biomagnification takes place when the concentration of a chemical increases along the food chain [3]. Due to the high stability and high solubility of chlorinated hydrocarbons in lipids, DDT concentrations undergo a biomagnification process when moving from lower to higher trophic levels within the ecosystem [10]. For instance, in Michigan Lake ecosystem, the concentration of DDT in the herring gull was 22 times

that of fish, which in turn had concentrations 300 times that in muds [10]. Thus, when moving higher in the food chain from muds to fish and finally to herring gulls, there was a 7000-fold increase in the concentration of DDT. Similar findings were reported for PCBs [3, 8]. The concentration of PCBs in the eggs of herring gull in the Great Lakes was 50,000 times that of the phytoplankton in the water [3].

4. Transport in the environment

POPs are present in relevant amounts in different environmental media, including soil, sediment, water, and air, and they are reversibly exchanged between these different media [11].

Some of the PCBs and other POPs that are present in soil and water, volatilize and enter the air. Vaporized PCBs will be adsorbed on particulates, which will then be carried by wind and redeposited on land or water by sedimentation or rain-out [4]. Since PCBs are not very soluble in water, they tend to be adsorbed onto suspended particles in water [3]. Thus, the deep sediments of oceans and huge lakes are the final sinks for mobile PCBs [3].

5. Mechanism in the body

a. Distribution in different organs

When PCBs enter the human body, they are rapidly transported by blood through association with blood cells and plasma proteins to various tissues [12]. Partitioning of PCBs between blood and tissues is determined by the lipid content and concentration gradient [12]. The main factors that affect the distribution of these

chemicals to various tissues are the volume, affinity, and rate of perfusion of the tissue [12].

i. Brain

Despite the high percentage of fat in the brain, PCBs are present at low concentrations in it [13]. The reason PCBs do not accumulate in the brain may be the blood-brain barrier that blocks the passage of PCBs to the brain [13].

ii. Lungs

Low-chlorinated PCBs are present at high concentrations in the lungs [13]. Since low-chlorinated PCBs have higher vapor pressure than high-chlorinated PCBs, they are volatilized into the air, and inhalation from the atmosphere introduces these chemicals to the lungs. Moreover, the accumulation of low-chlorinated PCBs in the lungs may be due to the presence of binding proteins specific for these compounds [13]. For instance, a binding protein in the human airway for methylsulfonyl metabolites of PCBs has already been confirmed in a previous study [14].

iii. Muscle tissue

High-chlorinated biphenyls enter the body through the ingestion of PCB contaminated foodstuffs of animal origin. High concentrations of these chemicals are found in the muscle tissue fat [13]. Muscle, along with liver, is a primary early depot for dietary PCB contaminants due to its large volume [12].

iv. Adipose tissue

Since PCBs are highly soluble in lipids, they have high affinity to lipid-rich tissues, such as adipose tissue [12]. Thus, PCBs are mainly concentrated in adipose tissue and skin [12].

b. Metabolism

The number and position of chlorine atoms within the PCB molecule determine the rate and degree of metabolism of PCBs [15]. In general, as the number of chlorine atoms on the biphenyl increases, the rate of metabolism decreases [16]. Thus, low-chlorinated PCBs are more susceptible for metabolic conversion compared to the high-chlorinated PCBs. In addition to the low number of chlorine atoms, the metabolism of PCBs is facilitated when the two adjacent carbon atoms in the meta and para positions are unsubstituted, since they promote the formation of arene oxide intermediate [15]. Examples of PCB metabolites include hydroxylated PCBs, PCB methyl sulfones, sulfates and glucuronides. The major metabolites of PCBs are the hydroxylated PCBs, which are formed through the oxidation of PCBs by cytochrome P-450 enzyme either via the formation of arene oxide intermediate or by direct insertion of hydroxyl groups [12, 17]. Depending on their structure, OH-PCBs are either metabolized and excreted or they are retained in the body, mainly in the blood [16]. For instance, sulfation could be a metabolic pathway for low-chlorinated OH-PCBs [16], knowing that OH-PCBs, depending on their chlorine substituents, are either substrates or inhibitors for sulfotransferases [18]. In contrast to OH-PCBs that are polar, $MeSO_2$ PCBs are lipophilic and thus they accumulate in the liver and lung [16].

c. Excretion

All biological excretory mechanisms are polar systems [12]. Since PCBs are not soluble in water, they are not readily excreted [12]. In order to be excreted, PCBs must be metabolized first into more polar compounds, such as OH-PCBs [12].

d. Elimination

In contrast to excretion, which is an active process, elimination is passive. PCBs can be eliminated, without being metabolized, by substances having the greatest volume and/or lipid content, such as eggs, milk, and fetuses [12].

6. In Lebanon

Lebanon banned OCPs in 1982 and PCBs in 1997 and adopted the Stockholm Convention on Persistent Organic Pollutants [5]. Although these pollutants were banned by the Lebanese government, we are still continually being exposed to them, mainly through the food chain: fish, meat and dairy products. Consequently, PCBs and OCPs are still being detected in different environmental media, such as surface water, ground water, soil, and sediments. In addition, they continue to be present in biological samples, such as human milk and serum. In addition to their persistence in the environment, they are still present due to unauthorized discharge into the rivers or illegal use of POPs despite their ban [5]. Several studies show that DDT and its metabolite DDE were the most abundant OCPs detected in Lebanese rivers, groundwater, and sediments [19]. For instance, DDE and β-endosulfan were detected at measurable levels in Hasbani River, Ibrahim River and Litani River [20-22]; DDE and HCB were the most frequently detected pesticides in both surface and groundwater

[23]; PCB-101, PCB-138, and PCB-153 were present in sediment samples in Tripoli Harbor [24]. Moreover, among the PCB congeners, PCB-138, PCB-153 and PCB-180 were the most frequently detected in human serum [19]. Finally, DDT and its metabolites were the most common OCP residues found in human milk [25].

B. Obesity

Obesity is a major risk factor for noncommunicable diseases (NCDs), such as cardiovascular diseases, diabetes, musculoskeletal disorders, and some cancers [26]. According to WHO, over 600 million adults were obese in 2016 [26]. In addition, obesity and overweight result in 3.4 million deaths yearly [27]. In Lebanon, NCDs were estimated to account for 91% of all deaths in 2016 [28]. Moreover, the rate of obesity in Lebanon follows an increasing trend. For instance, the percentages of the population who were obese in year 2000 were 18% and 28% for males and females, respectively. These percentages increased to 27% and 35% in 2015 [28].

Although there are many factors that can lead to obesity, such as unhealthy diet, lack of physical activity, sleep deprivation, and genetic factors, these alone cannot explain the increasing and high rates of obesity worldwide [29]. Environmental exposure to POPs adds another risk factor for obesity, since they are known to be endocrine disrupting chemicals that interfere with metabolism and disrupt normal homeostatic controls over adipogenesis and energy balance [29, 30].

C. POPs and Obesity

1. POPs as Endocrine Disrupting Chemicals

PCBs are endocrine disrupting chemicals, since they play a role in the disruption of thyroid homeostasis. Some studies demonstrate an inverse relationship between PCB levels, particularly OH-PCBs, and thyroid hormone levels [31]. One possible suggestion is that PCBs, due to their high affinity for thyroid hormone transport protein (TTR), displace pro-hormone L-thyroxine (T_4) from its binding sites on the TTR, thus leading to low thyroid hormone levels [32]. Since thyroid hormone regulates metabolism and cholesterol synthesis, hypothyroidism might lead to weight gain through reduction of metabolism and lipolysis [29, 33]. In addition to the OH-PCBs, $MeSO_2$-PCBs and PCB sulfates are also known to affect thyroid hormone homeostasis though reduction of serum T_4 levels [34] and binding with high affinity to TTR [35], respectively.

Other than the thyroid system, PCBs affect the endocrine system through their interaction with the estrogen receptor [36]. For instance, low-chlorinated OH-PCBs are known to be estrogenic, while antiestrogenic behavior has been reported for higher chlorinated OH-PCBs [36, 37].

OCPs are also known to be endocrine disrupting chemicals. For instance, p,p'-DDT and its metabolite p,p'-DDE exhibit both estrogenic and antiandrogenic activities [38].

2. Mechanism of POPs leading to obesity

In animal studies, exposure to POPs has resulted in reductions in energy expenditure and thermogenesis, indicating metabolic disruption associated with obesity [39]. Impaired adipogenesis in peripheral subcutaneous adipose tissue, disruption of

lipid metabolism, alterations in energy balance, and tissue lipid accumulation are potential causes of obesity [40].

The exact molecular mechanism for obesogenic compounds is still unknown [41]. One proposed mechanism includes alteration of gene expression after binding to the aryl hydrocarbon receptor, peroxisome proliferator activated receptor gamma (PPARγ) and estrogen receptors (ERs) [41]. PPARγ are nuclear receptors found in adipocytes and are considered to be a master regulator of adipogenesis and lipid homeostasis [39, 40]. Binding of obesogens, such as POPs to these receptors might promote adipogenesis, thus leading to obesity.

3. Prenatal exposure to POPs and fetal growth

POPs are transferred from mothers to infants through many routes. For instance, PCBs and OH-PCBs were found in both maternal and cord blood, which indicate that these chemicals can cross the placenta and reach the fetus [42]. In addition, one of the elimination routes for POPs is through breastmilk, as mentioned previously. Thus, breastfeeding is also responsible for the transfer of OCPs and PCBs to the infant [43]. The developing fetus is the most vulnerable to these chemicals, since the blood-brain barrier, liver metabolism, DNA repair mechanisms, immune system, detoxifying enzymes, and other protective mechanisms are not fully functional or developed [44]. Moreover, the impact of in utero exposure to POPs on physical growth, including birth weight, BMI, motor development, and short-term memory, persists from infancy to childhood [45]. For instance, low birth weight as a result of POPs exposure in early childhood is associated with adiposity in adolescents and earlier pubertal maturation, and is a significant predictor of the development of obesity and other diseases in adults,

such as cardiovascular diseases and hypertension [46]. A crucial phenomenon that relates low birth weight and fetal growth restriction with the development of obesity later in adulthood is “adiposity rebound”. Adiposity rebound corresponds to the second rise in the BMI between ages 5 and 7 years after it reaches a minimum level [47]. Prenatal PCB and DDE exposure was indeed associated with a higher change in BMI from 5 to 7 years of age, which may be linked to an early adiposity rebound [48]. Early adiposity rebound is associated with high fatness after the rebound and an increased risk of obesity in adolescence and adulthood [47]. Thus, intrauterine exposure to these chemicals might lead to the development of obesity in children and consequently in adults.

D. Objective of the study

Since prenatal exposure to POPs could have the greatest impact on future weight gain, we performed a systematic review and meta-analysis to investigate the associations between prenatal exposure to PCBs and DDE and childhood obesity. Next, we developed an analytical method that can be used to measure PCB and DDE concentrations in the serum samples collected from the cord blood and maternal blood.

CHAPTER II

PRENATAL EXPOSURE TO PCBS AND DDE AND CHILDHOOD OBESITY: A SYSTEMATIC REVIEW AND META-ANALYSIS

This review is part of a big systematic review that aims to investigate the associations between exposure to POPs and body weight status among all age categories. Thus, the keywords initially entered included all POPs. In addition, the screening process was done based on the big research question. Among the included studies after screening, only studies related to prenatal exposure to PCBs and DDE assessed in maternal serum or cord blood and childhood BMI z-score and/or risk ratio for overweight were included in this systematic review and meta-analysis.

A. Methodology

1. Search strategy

The search was conducted using four databases: Medline, Cochrane, Excerpta Medica Database (Embase), and Cumulative Index of Nursing and Allied Health Literature (CINAHL). All keywords related to the field of 'persistent organic pollutants' and 'weight or adipose tissues' were included. The final search was conducted on March 20, 2023.

2. Inclusion/exclusion criteria

Studies were included in this review if they met the following criteria: studies containing original research data that are peer-reviewed, of observational or

interventional designs, with children as a population of interest, assessing the prenatal exposure of PCBs and DDE in maternal serum or cord blood, and evaluating BMI z-score and overweight status as the outcomes of interest.

The exclusion criteria were the following: case reports and case series, narrative and systematic reviews, qualitative studies, studies that do not assess the exposure to PCBs and DDE and the outcomes quantitatively, studies written in Chinese, Japanese, or Korean languages, and studies with more than 25% of the population suffering from chronic diseases that affect body weight status.

3. Data extraction

For each article that met the inclusion criteria after abstract and full text screening, the following information were extracted: first author, year of publication, age and gender of children, exposure, unit of exposure, exposure assessment: matrix and time the sample was taken from mothers or infants, median or geometric mean of exposure, outcome: adjusted linear regression coefficients beta for the BMI z-score as continuous variable with their 95% confidence intervals (95% CI) or standard errors (SE) and adjusted relative risk ratios (RR) for the overweight status with their 95% CI or SE, and results.

4. Data synthesis and meta-analysis

After the data were extracted, meta-synthesis was done using RevMan software. For each outcome (BMI z-score and overweight status) and each exposure (DDE and PCB), a random effects meta-analysis was performed with the studies having the same

exposure unit when at least two studies were available. Heterogeneity between studies was evaluated using I^2 and significance was assessed using the p-value.

B. Results

1. Included studies

After entering the keywords on the four databases, 22,428 references were obtained. Removing the duplicates resulted in 16,591 references. These articles were screened first by title and abstract. 13,332 references were excluded, leaving 3,259 references for full-text screening. Among these references, 316 studies could not be retrieved due to full-text unavailability. From the remaining 2,943 references, 13 studies were included in this systematic review and meta-analysis. The screening process is illustrated by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram shown in figure 2.

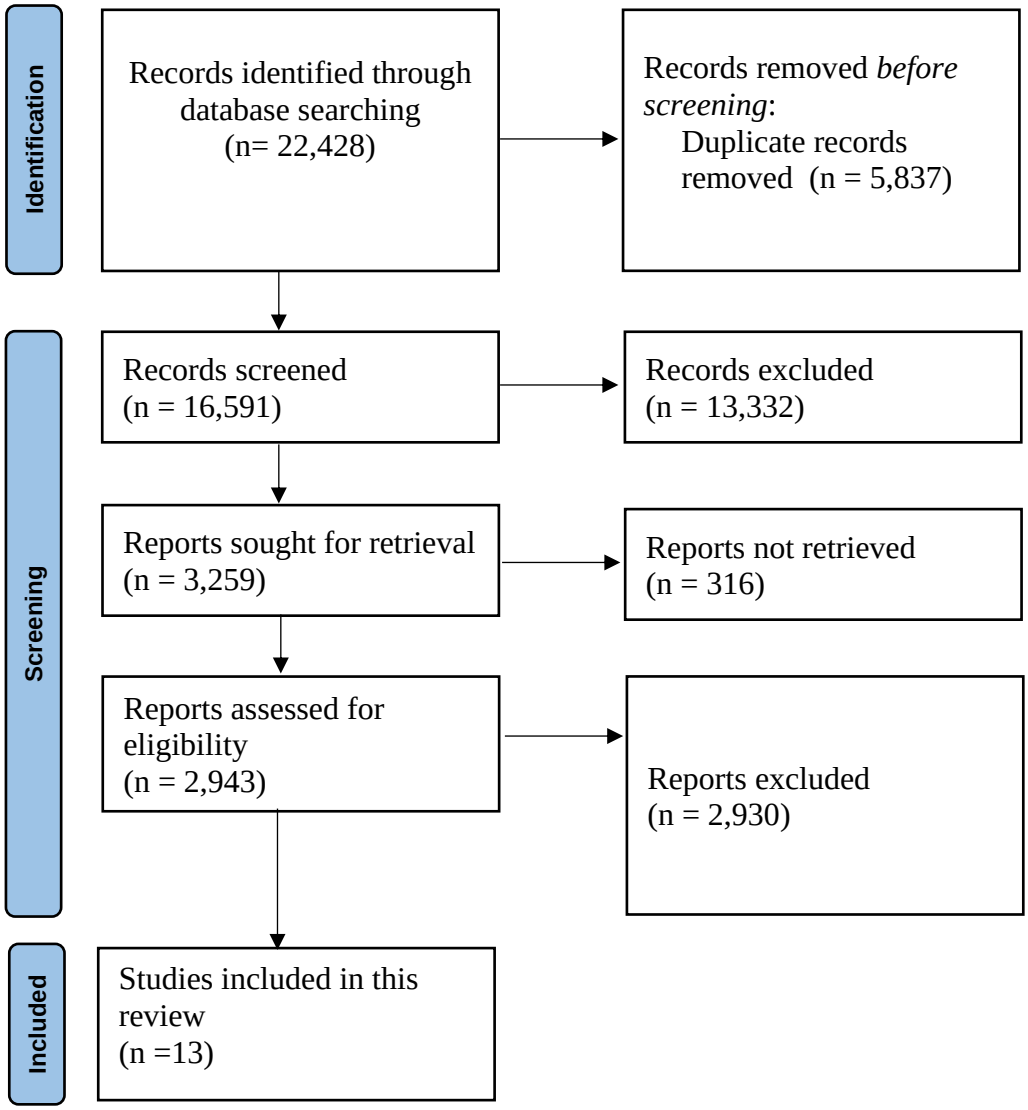

Figure 2: PRISMA flow diagram of the screening process

2. Characteristics of the studies

The characteristics of the studies included in this review are presented in table 1. The age of the children was between 1 and 12 years, thus covering the childhood period. The exposure to PCBs and DDE was assessed mainly in maternal serum collected during pregnancy or immediately after delivery or in cord blood collected at delivery. The median or geometric mean of exposure for the wet-based values was

between 0.24 ng/mL and 6.6 ng/mL for DDE and between 0.32 ng/mL and 0.71 ng/mL for PCBs.

Author	Year	Age	Exposure	Unit of exposure	Exposure Assessment	Median or geometric mean of exposure	Outcome	Results
Güil-Oumrait [49]	2021	6 years	p,p'-DDE and ΣPCBs	log_{10}(ng/mL)	cord blood	DDE: 1.68 ng/mL; PCBs: 0.71 ng/mL	BMI	DDE and PCBs were not significantly associated with BMI
Yang [50]	2021	2 years	p,p'-DDE	ln(ng/g)	cord serum	31.92 ng/g	BMI and overweight	no significant relationship was observed between DDE exposure and obesity status
Huang [51]	2018	3.5 years	p,p'-DDE	log_{10}(ng/g)	maternal serum collected prior or immediately after delivery	252.4 ng/g	BMI	DDE exposure was not strongly associated with BMI
Coker [52]	2018	1-2 years	p,p'-DDE	log_{10}(ng/g)	maternal serum collected just before or soon after delivery	285.52 ng/g	BMI	Association between DDE and BMI was in the positive direction, but was not statistically significant; association was significantly positive for girls, but nonsiginificantly negative for boys
Lauritzen [53]	2018	5 years	DDE and PCB-153	ln(ng/g)	maternal serum collected in the second trimester	DDE: 211 ng/g; PCB: 79.9ng/g	BMI	An increase in PCB concentration was associated with a nonsignificant increase in BMI
Warner [54]	2017	12 years	p,p'-DDE	log_{10}(ng/g)	maternal serum collected during pregnancy	1129.2 ng/g	BMI and overweight	An increase in DDE concentration was significantly associated with an increase in BMI and obesity risk in boys, but not in girls
Karlsen [55]	2017	5 years	DDE and ΣPCBs	log_{10}(ng/g)	2-week postpartum maternal serum	DDE: 0.13 μg/g; PCBs: 0.42 μg/g	BMI and overweight	DDE and PCBs were not significantly associated with BMI or risk for overweight

Vafeiadi [56]	2015	4 years	DDE and ΣPCBs	log_{10}(pg/mL)	first-trimester maternal serum	DDE:2036.2 pg/mL; PCBs: 319.1 pg/mL	BMI	DDE was significantly associated with an increase in BMI; PCBs were positively but not significantly associated with BMI
Valvi [57]	2014	14 months	p,p'-DDE and ΣPCBs	log_{10}(ng/g)	maternal serum collected in the first trimester of pregnancy	DDE: 132 ng/g; PCBs: 93.2 ng/g	overweight	DDE exposure was associated with overweight; exposure to PCBs was not associated with overweight
Delvaux [58]	2014	7-9 years	p,p'-DDE	ln(ng/mL)	cord blood	0.24 ng/mL	BMI	DDE exposure was positively, but nonsignificantly associated with BMI
Warner [59]	2014	9 years	p,p'-DDE	log_{10}(ng/g)	maternal serum collected during pregnancy	1104 ng/g	BMI	An increase in DDE concentration was associated with an increase in BMI in boys, but not in girls
Warner [60]	2013	7 years	p,p'-DDE	log_{10}(ng/g)	maternal serum collected during pregnancy	1422 ng/g	BMI	DDE exposure was nonsignificantly positively associated with BMI
Garced [61]	2012	1 year	p,p'-DDE	log_{2}(ng/mL)	serum samples were collected during each trimester of pregnancy	6.6 ng/mL	BMI	No significant association was found between DDE exposure and BMI

Table 1: The characteristics of the studies included in this review

3. Meta-analysis

Since only studies having the same unit for the regression coefficient can be pooled together and analyzed on RevMan, and since minimum two studies are required for meta-analysis, studies having the DDE and PCB concentrations expressed as ng/g and $\log_{10}$ transformed were pooled together and included in the meta-analyses.

Thus, three groups were entered into RevMan: DDE and BMI z-score, DDE and overweight status, and PCBs and overweight status.

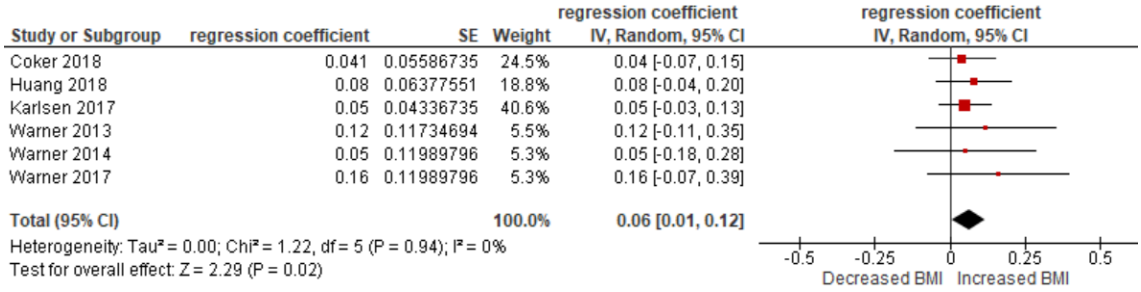

Figure 3: Forest plot displaying the association between prenatal exposure to DDE and childhood BMI z-score

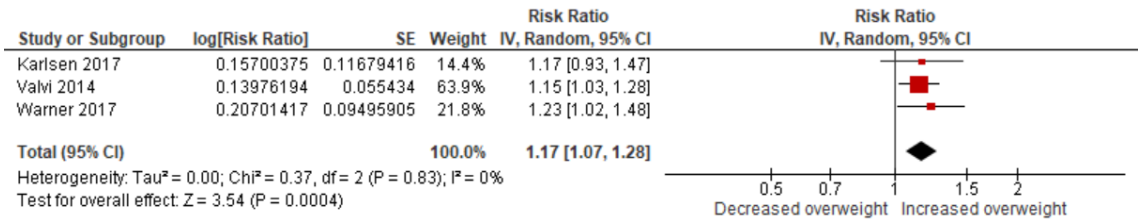

Figure 4: Forest plot displaying the association between prenatal exposure to DDE and childhood overweight risk

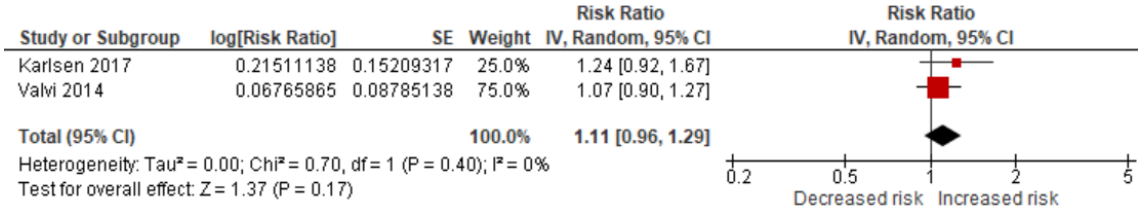

Figure 5: Forest plot displaying the association between prenatal exposure to PCBs and childhood overweight risk

C. Discussion

1. DDE and obesity

As shown in table 1, most of the studies included in this review indicated that the association between prenatal exposure to DDE and BMI z-score during childhood is in the positive direction. Some of these studies indicated that the increase in BMI and /or overweight risk as a result of DDE exposure is nonsignificant [49-53, 55, 58, 60, 61], while others reported a significant increase in BMI [56, 57]. Moreover, some studies provided sex-specific effects. Coker et al. reported that BMI z-score increased significantly as a result of DDE exposure in girls only [52], while Warner et al. indicated that an increase in DDE exposure was significantly associated with an increase in BMI and overweight status in boys only [54, 59]. These sex-specific associations might be due to the anti-androgenic and estrogenic activity of DDE, which both play key roles in adipogenesis during development [62]. Thus, intrauterine exposure to DDE may impact normal weight homeostasis either by directly affecting the deposition, differentiation, and metabolism of adipose tissue or by disrupting the endocrine feedback loop [62].

The results of the meta-analyses for the prenatal exposure to DDE and BMI z-score and overweight status are displayed in figures 3 and 4, respectively. As shown in

figure 3, the pooled regression coefficient is 0.06 (95% CI: 0.01, 0.12). Thus, a 10-fold increase in prenatal DDE exposure results in a significant increase of 0.06 BMI z-score, with a p-value of 0.02 and 0% heterogeneity (I^2=0%). This result is further confirmed by the meta-analysis of the overweight risk ratio. As displayed in figure 4, the pooled RR is 1.17 (95% CI: 1.07, 1.28), I^2=0% and p=0.0004. Since RR is greater than 1.00, this means that the risk of overweight is increased in the population exposed to DDE. Thus, the relation between prenatal DDE exposure and overweight status is significantly positive.

2. PCBs and obesity

Limited number of studies analyzed the association between prenatal exposure to PCBs and childhood BMI and overweight status. Most of these studies indicated a nonsignificant increase in BMI and/or overweight risk as a result of PCB exposure [53, 55-57]. This was confirmed by the results of the meta-analysis displayed in figure 5. The pooled RR being 1.11 (95% CI: 0.96, 1.29), indicates that prenatal exposure to PCBs increases the risk of overweight during childhood. However, the p-value is 0.17, which is greater than 0.05, this means that this association is not significant.

D. Conclusion

According to the data reported in the literature and the meta-analysis performed in this systematic review, prenatal exposure to DDE is associated with a significant increase in childhood BMI z-score and overweight status. Thus, these results support the chemical obesogen hypothesis, which states that intrauterine exposure to the endocrine disrupting chemicals, such as DDE, may increase risk of obesity later in life.

CHAPTER III

ANALYTICAL METHOD FOR THE QUANTIFICATION OF PERSISTENT ORGANIC POLLUTANTS IN SERUM

A. Chemicals and Reagents

A mixture containing different PCB congeners, which includes PCB-20, PCB-35, PCB-101, PCB-118, PCB-138, PCB-153 and PCB-180, dissolved in iso-octane, 2,4'-DDE solution in methanol, TCMX solution in methanol, 4,4'-DDE solid, and PCB-209 solid were purchased from Sigma-Aldrich. Formic acid, acetonitrile, HPLC grade water, and dichloromethane were purchased from Fisher Chemical. n-Hexane was obtained from Honeywell Riedel-de Haën. Methanol was obtained from Sigma-Aldrich.

B. Methodology

1. Preparation of standard solutions

PCB standard solutions, initially at a concentration rage of 5.9-10.5 μg/mL, were either used directly to prepare the high concentration samples for the calibration curve or were diluted ten times in hexane: DCM (9:1, v/v) in order to prepare the low concentration samples. 2,4'-DDE solution with initial concentration of 100 μg/mL was diluted several times in hexane: DCM (9:1, v/v) to reach a concentration of 5 μg/mL and 0.5 μg/mL, from which the high and low concentration samples were prepared, respectively. 4,4'-DDE was dissolved in hexane: DCM (9:1, v/v) to prepare a 500 μg/mL solution, which was then diluted several times in the same way as that of the 2,4'-DDE solution. TCMX (internal standard) solution initially at a concentration of 200 μg/mL was diluted ten times to a concentration of 20 μg/mL, from which a fixed

quantity (100 ng/mL) was added to all the samples. 1.26 mg of PCB-209 (internal standard) was dissolved in 1 mL hexane: DCM (9:1, v/v), which was then diluted hundred times to reach a final concentration of 12.6 μg/mL. A fixed quantity (100 ng/mL) of this PCB-209 solution was added to all the samples.

2. Sample pretreatment

Pooled serum initially stored at -20^{o}C was thawed at room temperature and vortexed to ensure that is homogenous. 1 mL pooled serum was added into a centrifuge tube and spiked with the standard (PCBs and DDEs) and internal standard (PCB-209 and TCMX) solutions.

To avoid interference from compounds present in serum, such as proteins that might coelute with the analytes of interest, the proteins must be precipitated first.

Different precipitating agents were tested. Among methanol, acetonitrile and acetonitrile plus formic acid, the best results were obtained with acetonitrile plus formic acid. The addition of organic solvent, such as acetonitrile, destroys the hydration layer of proteins, reduces the repulsive forces between them, and lowers the solubility of the proteins, resulting in their precipitation, while formic acid is an additive that reduces protein binding [63].

Thus, 1 mL formic acid (1%), 1mL acetonitrile and 1mL HPLC grade water were added to ensure protein precipitation and the mixture was vortexed for 1 min.

Precipitating agent	Result
Reference	peaks are not well separated (co-elution)
Methanol	low recoveries
ACN	more efficient protein precipitation + higher recoveries than methanol
ACN + formic acid	most efficient protein precipitation + highest recoveries

Table 2: The precipitating agents used and the results obtained by GC-MS analysis

3. Liquid-liquid extraction

The two most frequently used methods to extract POPs from serum are solid phase extraction (SPE) with C18 sorbent and liquid-liquid extraction (LLE). The latter was used with hexane: dichloromethane (9:1, v/v) as a solvent in order to avoid the problem of contamination of plastic coming from the SPE cartridges that might cause interfering peaks.

5 mL hexane: dichloromethane (9:1, v/v) was added to the serum samples containing the standard and internal standard solutions and the precipitating agents. Later, the samples were mixed in a shaker for 30 min and then centrifuged at 3000 rpm for 30 min. Then, the organic layer was transferred into a vial. The extraction process was repeated twice to ensure efficient extraction. The combined extracts were dried completely under a nitrogen stream and the dry residue was reconstituted in 150 μL hexane: dichloromethane (9:1, v/v). Finally, the sample was analyzed using GC-MS.

4. Instrumental analysis using GC-MS

Analysis of POPs was accomplished by Thermo Trace GC-Ultra Polaris ITQ 900 MS coupled with AS 3000 II autosampler. The separations were done using an Rxi-17Sil MS (30 m x 0.25 mm ID x 0.25 μm) column. The injection temperature was 280ºC. The samples (2μL) were injected in splitless mode. Helium was used as a carrier gas with a flow rate of 1 mL/min. The GC oven initial temperature was 80°C. Then, the temperature was increased to 220°C at 25°C/min, then to 245°C at 2°C /min, and then to the final temperature of 275ºC at 20°C /min rate that was held for 5 minutes. MS transfer line temperature was set at 270°C and ion source temperature at 250°C. Finally, single ion monitoring (SIM) mode was used as the scan mode to ensure higher sensitivity.

Compound	Retention time (min)	Quantifier Ion	Qualifier Ions
TCMX	5.93	207	209, 136
PCB-20	8.06	186	256, 188
PCB-35	9.22	186	256, 188
PCB-101	10.75	326	254, 324
2,4'-DDE	10.97	246	248, 176
4,4'-DDE	11.91	246	248, 176
PCB-118	13.20	326	254, 324
PCB-138	13.70	360	362, 290
PCB-153	15.59	360	362, 290
PCB-180	18.49	324	396, 394
PCB-209	24.41	178	356, 213

Table 3: The retention times, quantifier and qualifier ions for the analytes after GC-MS analysis

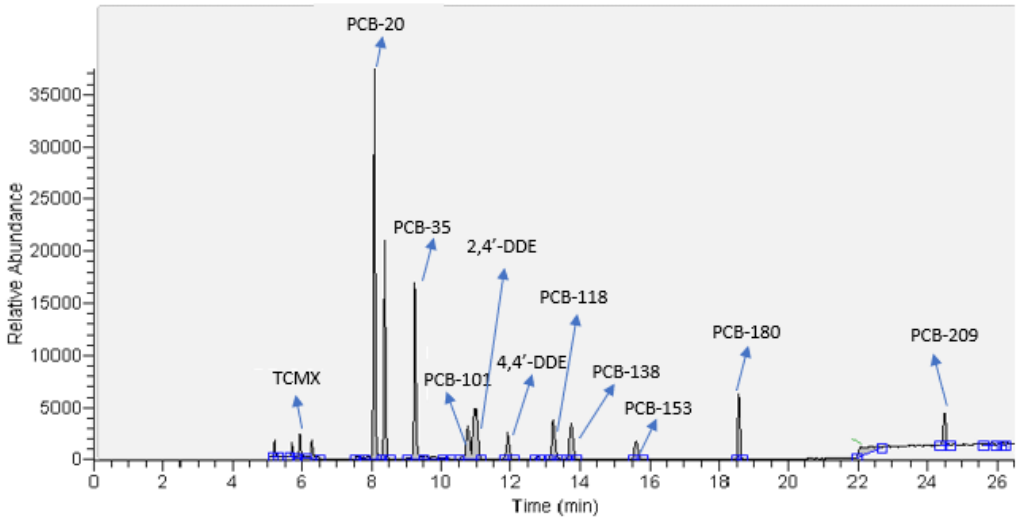

Figure 6: Chromatogram obtained by GC-MS analysis of the PCBs and OCPs

C. Validation of the method

1. Calibration curve

Calibration curve is a relationship between the response of an instrument and known concentrations of an analyte [64]. A calibration curve is constructed for each analyte in the sample [64]. A minimum of five points are needed to construct the calibration curve.

The ratio standard/internal standard is used to construct the curve and facilitate the quantification of the analyte. An internal standard is a compound different from the analyte that is added at known concentration to both the samples that are used to prepare the calibration curve and to the unknown samples [65]. It is important to add an internal standard when the instrument response varies slightly from run to run, especially when using GC, or when sample loss occurs during sample preparation [65].

PCB-209 was used for the quantification of the PCBs and TCMX was used for the DDE.

Limit of detection, LOD, is the lowest concentration of an analyte that can be differentiated from the background noise [64]. It can be determined based on the formula LOD=3s/m, where s is standard deviation of the lowest concentration sample and m is the slope of the calibration curve.

Limit of quantification is the lowest concentration of an analyte that can be quantified with acceptable precision and accuracy [64]. It can be determined based on the formula LOQ=10s/m.

Compound	PCB-20	PCB-35	PCB-101	2,4'-DDE	4,4'-DDE	PCB-118	PCB-138	PCB-153	PCB-180
Concentration (ng/mL)	5.6	5.23	5.28	5	5	5.49	3.45	5.23	5.55
	14	13.067	13.2	10	10	13.73	7.867	13.067	13.867
	28	26.13	26.4	20	20	27.467	15.73	26.13	27.73
	70	65.33	66	50	50	68.68	39.33	65.33	69.33
	140	130.6	132	100	100	137.3	78.6	130.6	138.6

Table 4: The concentrations of the analytes used to construct the calibration curve

2. Selectivity

Selectivity is the ability of an analytical method to distinguish the analytes from all other components that may be present in the sample [64]. Based on the chromatogram obtained by GC-MS analysis of POPs, all the analytes were baseline separated without any coelution or interference.

3. Precision

Precision describes the closeness of individual measures of an analyte when the same analytical procedure is applied several times [64]. It is calculated based on the

relative standard deviation: %RSD= (standard deviation/average) *100. For acceptable precision %RSD must be less than 20% [64]. Two types of precision are tested: intraday and inter-day.

Intraday precision was determined based on the preparation of 4 samples at each concentration (low, medium and high) during the same day.

Inter-day precision was determined based on the preparation of 4 samples at each concentration (low, medium and high) at 3 days.

4. Recovery

Recovery of an analyte is determined by comparing the detector response obtained from an amount of analyte added to and extracted from the biological matrix and that obtained from the pure authentic standard [64]. It is an indication of the extraction efficiency of the analytical method [64]. Recovery should be determined for extracted samples at three concentrations (low, medium, and high) [64].

Compound	PCB-20	PCB-35	PCB-101	2,4'-DDE	4,4'-DDE	PCB-118	PCB-138	PCB-153	PCB-180
LOD (ng/mL)	1.7	1.12	1.51	1.48	1.4	1.14	1.84	1.84	1.37
LOQ (ng/mL)	5.67	3.73	5.02	4.93	4.68	3.82	6.14	6.14	4.56
Concentration	Intraday % Precision (Repeatability)								
Low	5.46	2.31	11.12	7.27	7.71	3.75	3.74	13.31	4.38
Medium	5.60	9.49	12.36	2.62	7.41	6.32	4.94	8.41	8.85
High	5.90	7.98	9.48	7.76	10.05	7.45	5.49	6.80	6.91
Concentration	Inter-day % Precision (Reproducibility)								
Low	2.65	5.57	6.86	21.65	7.84	8.38	13.10	13.45	6.67
Medium	5.67	10.86	14.90	8.34	12.11	2.30	6.32	5.61	2.92
High	14.18	20.89	7.71	9.03	5.50	9.09	7.01	5.19	12.45
Concentration	% Recovery								
Low	83.80	83.78	81.12	87.48	134.41	105.54	90.90	122.13	86.73
Medium	74.13	72.88	65.65	90.34	115.41	92.13	77.39	100.30	83.19
High	64.97	64.43	71.30	63.06	92.90	75.61	69.32	78.17	66.54

Table 5: Validation results of the PCBs and OCPs analysis

D. Results and Limitations

Due to the regulations placed on POPs, their levels are gradually declining in the environment. As a result, they are currently present at parts per trillion levels in human serum (as shown in table 1). Since the sensitivity of the GC available in the lab is not high enough, POPs were present below the detection limit in the serum samples of pregnant women obtained from AUBMC. Thus, due to the limitation of the instrument, POP levels could not be identified in real serum samples.

CHAPTER IV

QUANTIFICATION OF NICOTINE ENANTIOMERS IN ELECTRONIC CIGARETTES

A. Nicotine Enantiomers in Electronic Cigarettes

1. Electronic Cigarettes

a. Components and mode of action

Electronic cigarettes (ECIGs) are battery-powered devices, which imitate tobacco smoking by producing a heated vapor that looks like smoke [66]. These devices contain an electric heating element (atomizer) and an e-liquid that is stored in a cartridge [66]. The e-liquid is generally composed of propylene glycol, glycerin, nicotine, and flavoring agents [67]. When the atomizer vaporizes the e-liquid, an aerosol is generated, which is inhaled by the user [66].

b. Comparison with conventional cigarettes

i. Emissions

In contrast to the popular belief that ECIGs produce only harmless water vapor, studies show that there are some toxins emitted from ECIGs, but at levels much lower compared to conventional cigarettes. For instance, low levels of acetone, formaldehyde, acetic acid, isoprene, acetaldehyde, and 2-butanone were emitted from ECIGs [68]. However, emissions from conventional cigarettes included other toxicants as well, such as 1,2-propanediol, benzene, toluene, m,p-xylene, phenol, and 3-ethenyl-pyridine [68]. Another study showed that fine particles $PM_{2.5}$ were emitted from both ECIGs and conventional cigarettes, their mean concentration from tobacco cigarettes being seven

times higher than that from ECIGs [69]. Carbon monoxide (CO), being a key combustion element of conventional cigarette smoke, was not present in ECIG aerosol [69]. In contrast to CO, a rise of exhaled nitric oxide (NO) was observed after vaping nicotinic ECIGs [70]. Moreover, a study done on metals indicated that ECIG aerosols contained tin, silver, iron, nickel, aluminum, and chromium, at levels higher than or equal to those in conventional cigarette smoke [71]. These metals might have initiated from the wires and other metal components used in the atomizers of the ECIG devices [71].

Concerning the deposition of the emissions in organs inside the human body, such as alveoli, a study indicated that the total predicted deposition ranges from 20% to 27% for the ECIG aerosol, while slightly higher deposition is predicted for the cigarette smoke (25-35%) [72].

Thus, ECIG aerosols are not comprised of simply water vapor. Although the toxicants emitted are at a lower level compared to conventional cigarettes, the particles emitted are comparable.

ii. Health effects

Due to the toxicants emitted from the ECIGs, exposure to ECIG aerosol can result in biological effects. E-liquids are composed of propylene glycol and glycerin. When heated and vaporized, propylene glycol forms propylene oxide [73], while glycerin forms acrolein [74]. Propylene oxide is a probable human carcinogen, while acrolein vapor, at low exposure level, may cause eye, nasal, and respiratory tract irritations [75]. Moreover, the rise of exhaled nitric oxide as a result of ECIG aerosol exposure is associated with eosinophilic airway inflammation [70]. In addition, most of

the metals identified in ECIG aerosol are known to cause respiratory irritation and disease [71].

2. Nicotine

a. Properties

Nicotine, 3-[2-(N-methylpyrrolidinyl)]pyridine, is one of the main ingredients of cigarettes, making up about 95% of all the alkaloids in tobacco [76]. Nicotine is an oily, colorless hygroscopic liquid, which has a characteristic odor and turns brown upon exposure to air [77]. It is soluble in water and alcohol, having an octanol/water partition coefficient of 1.2 on the logarithmic scale [77]. In addition, it is a weak base, in which the pk_a of the pyridine nitrogen is 3.13 and that of the pyrrolidine nitrogen is 8.02 at 25^{o}C. Moreover, nicotine is an optically active chiral compound, which exists as two enantiomers, denoted by (S)-(-) and (R)-(+).

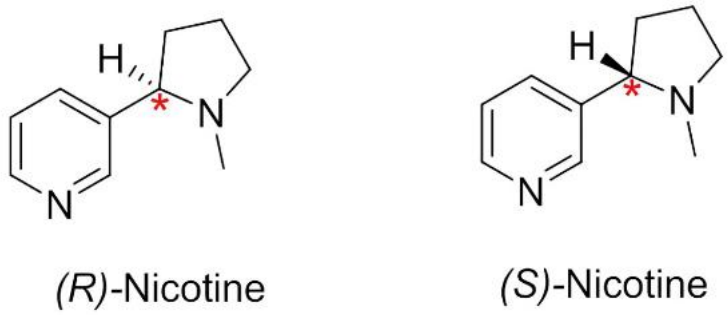

Figure 7: Structures of (R)-(+) and (S)-(-) nicotine

b. Absorption

Nicotine absorption through cell membranes depends on the pH [78]. In alkaline pH, nicotine exists mainly as the free base, facilitating its absorption through oral or nasal mucous membranes [79]. In general, as the pH of the cell membrane increases, the

rate of nicotine absorption increases. In acidic environments, nicotine is present mainly as the protonated form, preventing it from rapidly crossing membranes [77]. For instance, due to the low pH of the stomach, nicotine absorption through the gastric mucosa is poor [80]. On the other hand, nicotine is absorbed through the small intestine due to the alkaline pH and huge surface area [81]. The physiological pH of blood is 7.4, which is between the two pka values of nicotine (8.02 and 3.13). Since 31% of nicotine is present as free base, nicotine at this pH rapidly crosses membranes [79]. When nicotine reaches the lung, it is rapidly absorbed through the alveoli due to its huge surface area and due to the dissolution of nicotine in fluid of physiological pH (7.4) [77]. Moreover, nicotine can be absorbed through the skin [82].

c. Distribution in the body

After nicotine is absorbed, it enters the bloodstream and is distributed extensively to body tissues [83]. Spleen, liver, lungs, and brain have high affinity for nicotine, while the affinity of adipose tissue is relatively low [83]. Nicotine readily crosses the blood-brain barrier and enters the brain [84]. Moreover, elevated nicotine concentrations were found in amniotic fluid and umbilical vein serum, indicating that nicotine readily crosses the placenta [85].

d. Metabolism

Nicotine is extensively metabolized mainly in the liver, but also to a lesser extent in the lung and kidney [86]. The major metabolites of nicotine are cotinine and nicotine-N-oxide [83]. About 80% of nicotine is metabolized to cotinine [83]. The first step in the formation of cotinine from nicotine involves oxidation of the alpha carbon of

the pyrrolidine ring via cytochrome P450 enzyme to 5'-hydroxynicotine, which is in equilibrium with nicotine $\Delta^{1'(5')}$ iminium ion [87]. The second step involves metabolization of nicotine $\Delta^{1'(5')}$ iminium ion to cotinine by the cytoplasmic enzyme aldehyde oxidase [88]. Cotinine itself is also metabolized. Trans-3'-hydroxycotinine, cotinine-N-oxide, and 5'-hydroxycotinine are among the cotinine metabolites detected in human urine [79]. Nicotine is also metabolized to nicotine-N-glucuronide and several other minor metabolites [89].

e. Excretion

Renal excretion is a minor pathway for the elimination of unchanged nicotine, since most nicotine is metabolized extensively before being excreted [83]. The rate of nicotine excretion depends on urinary pH and urine flow [90]. In acidic urine, nicotine is mainly protonated, thus less nicotine is reabsorbed, and more nicotine is excreted. However, in alkaline urine, nicotine is in the free base form, thus it is reabsorbed, having low renal clearance rate [90].

f. Health effects

Nicotine can have adverse health effects on human health. It has been associated with cancer, cardiovascular diseases, respiratory disorders, gastrointestinal disorders, and reproductive problems.

Nicotine can cause increased cell proliferation and DNA mutations, thus promoting carcinogenesis [91]. In addition, nitrosation products of nicotine, such as N-nitrosonornicotine and nicotine-derived nitrosamine ketone, may increase lung cancer risk [92]. Nicotine contributes also to the development of cardiovascular disease, since

it increases heart rate, blood pressure, and cardiac contractility [91]. Moreover, nicotine causes an increased airway resistance by bronchoconstriction, which might lead to several respiratory disorders, such as chronic obstructive pulmonary disease [93]. In addition, nicotine has harmful effects on the gastric mucosa, since it increases acid, pepsin, and vasopressin secretions [94]. Finally, the effects of nicotine on the reproductive system are evident by the disruption of spermatogenesis [95] and the reduction in both oviductal and uterine blood flow, resulting in alterations in conceptus growth [96].

3. (R)-nicotine versus (S)-nicotine

a. Tobacco derived versus synthetic nicotine

Naturally, nicotine is mainly found as the (S)-enantiomer in tobacco plants (>99%), with very small amounts of the (R)-enantiomer (<1%) [97]. On the other hand, synthetic nicotine is usually produced as a racemic mixture 50:50 of (R) and (S) enantiomers, which can then be resolved to obtain enantiomerically pure (S)-nicotine and (R)-nicotine [98, 99].

b. Toxicity

Several studies indicate that the pharmacological effects of the two nicotine enantiomers are similar, but (R)-nicotine is less potent compared to (S)-nicotine. For instance, the intravenous lethal dose LD_{50}, which is the dose that proves to be lethal for 50% of the population of test animals [3], was found to be 0.33 mg/kg for (S)-nicotine and 6.15 mg/kg for (R)-nicotine [100]. Since chemicals having smaller value of LD_{50} are more potent, (S)-nicotine is expected to be more toxic than its antipode. In addition,

the relative potency of (R)-nicotine was approximately 0.06 that of (S)-nicotine in the case of elevation of rat blood pressure, 0.2 in the cat superior cervical ganglion stimulation and blockade, and 1.0 in the neuromuscular junctions of the rat diaphragm [101].

Oxidative stress can be estimated by both glutathione (GSH) and malondialdehyde (MDA) levels. Glutathione (GSH) protects cell membranes against peroxides and free radicals [102]. Free radicals generate lipid peroxidation, which results in MDA production [103]. A study done on Chinese hamster ovary cells showed that exposure to nicotine enantiomers imposed oxidative stress, which was evident by the decrease in GSH levels and the increase in MDA levels, demonstrating the generation of free radicals [104]. Another in vitro study done on human whole blood indicated an inhibition of thromboxane B_2 formation and reduction of leukotriene E_4 production as a result of exposure to nicotine enantiomers [105]. Leukotrienes are lipid mediators of inflammation and they play an important role in mediating immediate hypersensitivity reactions, such as asthma [106], while inhibition of thromboxane generation is associated with an increased risk of cardiovascular diseases [107]

The metabolic rate of (R)-nicotine is 1.4-fold faster than (S)-nicotine [108]. Experimental results indicated that (R)-nicotine was preferentially oxidized over (S)-nicotine by the cytochrome P-450 enzymes and theoretical free energy change calculations proved a 2.5-fold higher binding affinity to these enzymes for (R)-nicotine [108].

c. Applications

Despite the negative health effects of nicotine, studies show that nicotine can be used as a therapeutic agent for the treatment of neurodegenerative diseases.

Acetylcholine (ACh) is a molecule that acts as a neurotransmitter in the nervous system and it maintains homeostasis and brain functions [109]. Nicotinic acetylcholine receptors (nAChRs) are a class of ACh that induce rapid depolarization responses to stimulate neuronal excitation [109]. In addition, these receptors regulate memory, addiction, and dopamine release in the striatum.

Glutamate toxicity is one of the risk factors for neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [109]. nAChRs play a protective role in glutamate toxicity [109]. Since nicotine is an agonist of nAChRs, it could prevent glutamate toxicity by exerting a protective action against NMDA receptor-mediated intracellular responses to stimulate neuronal death [109].

Studies show that the affinity of the (R)-enantiomer of nicotine to nAChRs is about ten to hundred times lower than that of the (S)-enantiomer [110]. Since (R)-nicotine has a moderate affinity to nAChRs and lower toxicity compared to (S)-nicotine, it might be a useful therapeutic agent for the treatment of neurodegenerative diseases and tobacco smoking addiction [89].

4. Objective of the study

Since 2016, the Tobacco Control Act provided the Food and Drug Administration (FDA) authority to regulate cigarettes, cigarette tobacco, roll-your-own tobacco, smokeless tobacco, and any other tobacco products [111]. In order to keep their products on the market, manufacturers of electronic cigarettes started using

synthetic nicotine instead of the banned tobacco-derived nicotine [97]. However, synthetic nicotine is much more expensive than tobacco-derived nicotine [97]. Thus, it is possible that they might be claiming that their products contained synthetic nicotine, while actually using the much cheaper tobacco-derived nicotine or a mixture of the two [97]. For this reason, it is of great importance to find an analytical method that can distinguish the two types of nicotine. Developing an analytical method that can separate nicotine enantiomers can solve this issue. For instance, if (R)-nicotine is present at amounts greater than 1.5%, then the nicotine cannot be tobacco-derived.

Moreover, since nicotine enantiomers differ in the degree of potency, it is essential to determine the enantiomeric purity of e-liquids and aerosols emitted from them, for pharmacological and toxicological implications.

Thus, an analytical method will be developed that can separate nicotine enantiomers by HPLC-DAD. This method will then be used to quantify the (R) and (S) enantiomers of nicotine in e-liquids and aerosols from several ECIG brands.

B. Analytical Method Development, Optimization, and Validation

1. Chemicals and reagents

Pure (S)-(-)-nicotine was purchased from Sigma-Aldrich. Pure (R)-(+)-nicotine and pure racemic mixture of (R) and (S)-nicotine were purchased from Biosynth. Ammonium formate, methanol, vegetable glycerin, and propylene glycol were obtained from Sigma-Aldrich. Isopropanol, acetonitrile and HPLC grade water were purchased from Fisher Chemical. Ammonium hydroxide was purchased from Fisher Scientific. Ammonium acetate was obtained from Spectrum Chemical.

2. Methodology

a. Preparation of standard solutions

1000 μg/mL of (R)-(+) and (S)-(-) nicotine racemic mixture in isopropanol was prepared from pure nicotine standard solution. Starting from this solution, different volumes were taken to prepare solutions of different concentrations, which were then used to construct the calibration curve.

b. Optimization of the HPLC-DAD parameters for the enantioseparation

Among the methods used to separate nicotine enantiomers is chiral gas chromatography [112]. However, this method is not suitable, since the retention time is usually long and complete peak separation is not observed [112]. Another method reported in the literature is using microcolumn liquid chromatography with chiral β-cyclodextrin mobile phase [113]. This method is also not preferrable, since the analysis time is in hours. The best method used for the enatioseparation of nicotine is liquid chromatography with chiral stationary phase [114].

Thus, analysis of nicotine enantiomers was done by Agilent 1100 high performance liquid chromatography−diode array detection system (HPLC-DAD). The CHIRALPAK AGP column (15 cm x 4 mm column with 5 μm particle size) was used.

In order to achieve enantioseparation, the mobile phase, which consists of the buffer solution and the organic modifier, must be optimized. The pH of the buffer solution was the most important parameter affecting the separation. At pH 5.80, no separation was observed. At pH 7.33, some separation was observed in the absence of the organic modifier, but the peaks were very broad. The best separation was achieved at pH 9.00, by the addition of ammonium hydroxide. Among isopropanol, acetonitrile

and methanol, the best results were obtained using isopropanol as an organic modifier. Two buffer solutions were tested: ammonium acetate and ammonium formate. Better separation was obtained when using ammonium formate. Concerning the concentration of the buffer solution, 20mM was better compared to the 10mM solution. Under these conditions, the best percentage of organic modifier to achieve baseline separation was 0.5%.

Thus, separation of the enantiomers of nicotine was achieved using an isocratic mobile phase program consisting of 99.5:0.5 (v/v) of 20 mM ammonium formate (pH=9.00) and isopropanol. The flow rate of the mobile phase was 0.8 mL/min, the sample injection volume was 5μL and the wavelength of the detector was 254 nm.

Buffer	Buffer concentration (mM)	Buffer pH	Organic Modifier	% of Organic Modifier	Result
Ammonium acetate	10	5.80	isopropanol	5	No separation
Ammonium acetate	10	5.80	isopropanol	10	No separation, narrow peak
Ammonium acetate	10	5.80	-	0	No separation, broad peak
Ammonium acetate	10	7.33	-	0	Some separation, broad peaks
Ammonium acetate	10	7.33	isopropanol	0.5	Very slight separation
Ammonium acetate	10	7.33	isopropanol	1	No separation
Ammonium acetate	10	9.00	-	0	Separation, not baseline, broad
Ammonium acetate	10	9.00	isopropanol	0.5	Better separation, not baseline
Ammonium acetate	10	9.00	isopropanol	1	Almost baseline, narrow peaks
Ammonium acetate	10	9.00	methanol	1	Separation but not baseline
Ammonium acetate	10	9.00	acetonitrile	1	Almost baseline separation
Ammonium acetate	20	9.00	isopropanol	1	Almost baseline separation
Ammonium formate	10	9.00	-	0	Almost baseline, very broad peaks
Ammonium formate	10	9.00	isopropanol	1	Baseline, narrow peaks, tailing
Ammonium formate	10	9.00	isopropanol	1.5	No baseline separation, narrow
Ammonium formate	10	9.00	methanol	1	Separation but not baseline
Ammonium formate	10	9.00	acetonitrile	1	Separation but not baseline
Ammonium formate	20	9.00	isopropanol	1	No baseline separation, narrow
Ammonium formate	20	9.00	isopropanol	0.5	Baseline separation + narrow peaks, Rs=2.6

Table 6: Optimization parameters of the solvent used for HPLC-DAD analysis of nicotine enantiomers

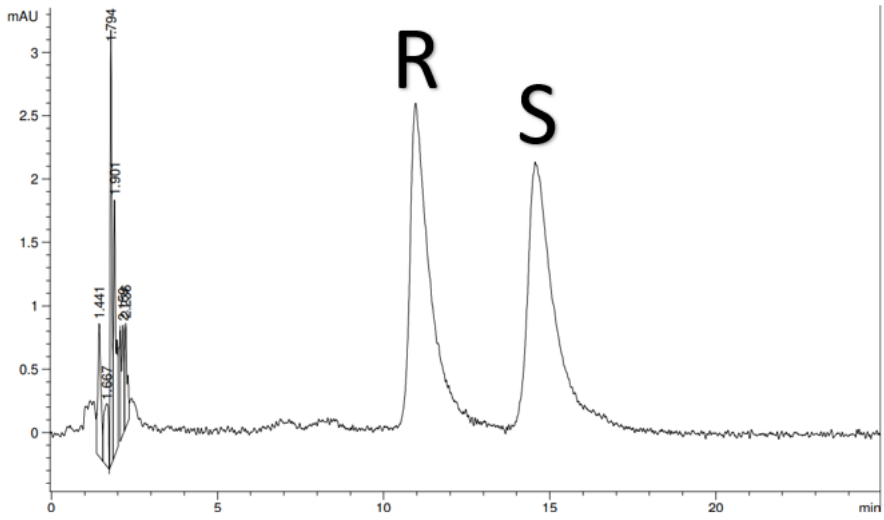

Figure 8: Chromatogram obtained by HPLC-DAD analysis of the nicotine enantiomers

c. Mechanism of enantioseparation

i. Role of the pH

Nicotine exists in three forms: diprotonated, monoprotonated and free base, depending on the pH of the medium.

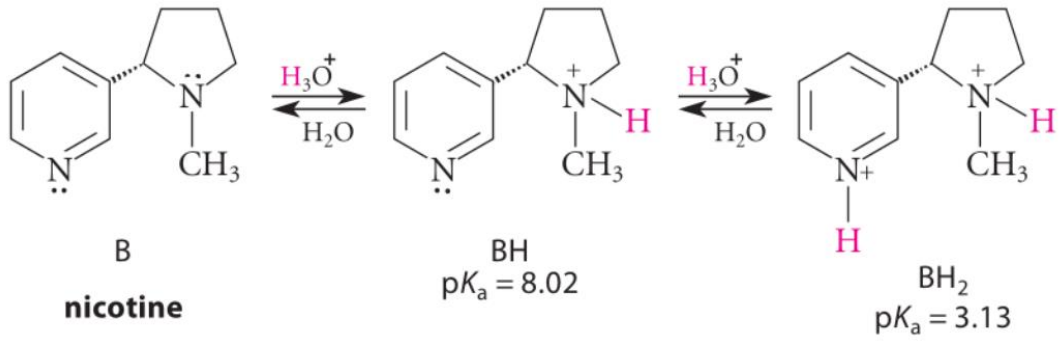

Figure 9: Free base, monoprotonated, and diprotonated nicotine

At pH 9.00, nicotine exists as the free base, since the pH is greater than the pka of nicotine (8.02). Since the enantioseparation is taking place at pH 9.00, where the main form of nicotine is free base nicotine, the interactions that are happening with the stationary phase are most probably hydrophobic, knowing that the α-acid glycoprotein has hydrophobic residues in its protein part, such as the amino acids lysine and tyrosine [115].

ii. Role of the column

Enantiomers have identical physical properties except the optical rotation, which is opposite for the two enantiomers. This means that, there is no chance to separate these enantiomers unless they are transformed into diastereomers, which have different physical properties, they do not have the same overall shape and they experience different intermolecular attractions. Thus, when the enantiomers interact with the chiral stationary phase, reversible diastereomer complexes are formed at the surface, which enable their separation [116].

iii. Role of the organic modifier

In the absence of organic modifier, the retention times were very long, and the peaks were very broad at all pH ranges and types of buffer solutions. Changing the percentage of the organic modifier affected greatly the resolution of the peaks. Baseline separation was achieved only at a specific percentage of organic modifier for each condition shown in table 6. Thus, the organic modifier played an important role in achieving baseline separation, while decreasing the retention time and making the peaks narrower at the same time.

d. Analysis of nicotine enantiomers in e-liquids

Due to the bitterness and harshness of free base nicotine, manufacturers started adding weak organic acids, such as benzoic, salicylic, and lactic acid to get the protonated nicotine salt, which improves the sensory experience of vaping and makes ECIGs more appealing [117]. However, in order to be able to analyze nicotine by HPLC-DAD, nicotine must be converted to one form. Since the pH of the mobile phase is 9.00, greater than the pka of nicotine, regardless of the initial form of nicotine, it is expected to be present in the form of free base at this pH.

In order to ensure efficient extraction of both free base and protonated nicotine from the e-liquids, two types of e-liquids were prepared. The first e-liquid contained free base nicotine, while the second protonated nicotine. The first e-liquid was prepared by adding propylene glycol and vegetable glycerin, 30/70 PG/VG, and 74 μL nicotine to a 5 mL volumetric flask to prepare 15 mg/mL nicotine mixture. Then, the mixture was vortexed and placed in a sonicator overnight to ensure it is homogenous. The second e-liquid was prepared using the same procedure as the first e-liquid, with the addition of 0.0565g benzoic acid to have protonated nicotine. After the mixtures became homogenous, they were transferred to a 10 mL vial.

50 μg/mL solutions were prepared by diluting 16.5 μL of the e-liquids in 5 mL isopropanol. Then, the samples were vortexed and placed on shaker for 15 min. Finally, 1 mL of each solution was transferred to a vial and analyzed using HPLC-DAD.

The results obtained by the HPLC-DAD analysis indicated that both free base and protonated nicotine behaved similarly, with greater than 95% recoveries for both types of e-liquids.

e. Aerosol generation from ECIG

ECIG aerosols were generated using the American University of Beirut's aerosol lab vaping instrument (ALVIN). Aerosols were trapped on two quartz fiber filters and one of these filters was taken for nicotine analysis. The number of puffs was 15, puff duration 4s and inter-puff interval 30s. These conditions were chosen to represent an ECIG user. The total particulate matter was determined gravimetrically by weighing the filters before and after each sampling session.

f. Analysis of nicotine enantiomers in ECIG aerosols

The analysis of nicotine in aerosols was done by adding 5 mL isopropanol to the vial containing the filter. The sample was placed on shaker for 30 min and then on sonicator for 10 min. Then, the sample was diluted five times in isopropanol and analyzed by HPLC-DAD.

3. Validation of the method

a. Calibration curve

Separate direct calibration curves were constructed for each of the (R) and (S) enantiomers of nicotine from standard nicotine solutions in isopropanol. The range of the calibration curve was from 2.5 μg/mL to 400 μg/mL. The limit of detection (LOD) was found to be 1.06 μg/mL for (R)-nicotine and 1.32 μg/mL for (S)-nicotine, while the limit of quantification was found to be 3.53 μg/mL for (R)-nicotine and 4.40 μg/mL for (S)-nicotine.

b. Repeatability

The repeatability of the method was determined based on the preparation of triplicate samples of e-liquids in isopropanol and was found to be within the acceptable range: %RSD for (R)-nicotine was 4.39% and for (S)-nicotine 3.40%.

c. Recovery

The recovery of the method was determined based on the preparation of triplicate samples of e-liquids in isopropanol. The recoveries were greater than 95% for all the samples.

C. Levels of Nicotine Enantiomers in Real ECIG Samples

1. Results

a. In e-liquids

The concentrations of (R) and (S) enantiomers of nicotine in Elf Bar, Lost Mary and NJOY e-liquid samples were determined by diluting 30 µL of e-liquid in 5 mL isopropanol based on the procedure described previously (refer to paragraph B.2.d.). The areas obtained by HPLC-DAD analysis were then substituted in the equations of the calibration curves to deduce the diluted concentrations of nicotine in µg/mL. Since the total volume was 5 mL, the mass of nicotine was obtained by multiplying the concentration deduced by 5. Finally, the initial concentration of nicotine in mg/mL was determined by dividing the mass of nicotine by 30.

brand	flavor	type of nicotine on label	nicotine content on label (mg/mL)	experimental total concentration of nicotine (mg/mL)	% (R)-nicotine	% (S)-nicotine
Lost Mary	Strawberry mango **(1)**	not specified	50	45.79	4.44	95.56
	Blue razz ice **(2)**	not specified	50	45.17	3.10	96.90
Elf Bar	Blue razz ice **(3)**	synthetic	50	44.38	0	100
	Watermelon ice **(4)**	synthetic	50	44.01	0	100
	Watermelon ice **(5)**	not specified	50	44.91	0	100
NJOY	Rich tobacco	not specified	69	65.21	0.51	99.49
	Classic tobacco	not specified	28	30.05	1.16	98.84
	Rich tobacco	not specified	51	49.37	0.68	99.32
	Menthol	not specified	51	49.47	0.81	99.19
	Menthol	not specified	68	69.80	0.80	99.20
	Classic tobacco	not specified	58	60.50	0.64	99.36
	Menthol	not specified	28	30.14	0.98	99.02
	Menthol	not specified	58	63.10	1.00	99.00

Table 7: Results obtained by HPLC-DAD analysis of nicotine enantiomers in e-liquids

b. <u>In ECIG aerosols</u>

The masses of total nicotine in the aerosols obtained by Elf Bar and Lost Mary ECIGs were determined based on the procedure described previously (refer to paragraph B.2.e.). The areas obtained by HPLC-DAD analysis were substituted in the equations of the calibration curves to obtain the concentrations in µg/mL in the diluted solutions. Since the dilution factor was 5, the initial concentration was obtained by multiplying the final concentrations by 5. The mass of nicotine in mg was determined by multiplying the final concentration by 5 and dividing by 1000, since the filters were placed in 5 mL isopropanol. These calculations were done for the first filters. Finally,

calculations were done using the total particulate matter for the first and second filters to obtain the mass of total nicotine per session.

nb	mass of nicotine (mg)	% (R)-nicotine	% (S)-nicotine
1	8.19	4.96	95.04
2	8.43	3.24	96.76
3	7.83	0	100
4	7.78	0	100
5	7.63	0	100

Table 8: Results obtained by HPLC-DAD analysis of nicotine enantiomers in ECIG aerosols

2. Discussion

a. <u>In e-liquids</u>

E-liquids 1 and 2 of Lost Mary contained (R)-nicotine at percentages greater than 1%, which indicates that the nicotine present is not completely natural, since natural nicotine exists as >99% (S)-nicotine. In addition, synthetic nicotine is usually produced as racemic mixture 50/50 of (R) and (S) nicotine, but can be resolved to obtain pure (S)-nicotine. Thus, Lost Mary e-liquids 1 and 2 contain either synthetic nicotine or a mixture of both synthetic and natural nicotine. On the other hand, all the other e-liquids most probably contain tobacco-derived nicotine, since the nicotine present is almost 100% (S)-nicotine. Thus, Elf bar e-liquids 3 and 4 that are claimed to contain synthetic nicotine might contain the cheaper natural nicotine, unless they resolved the synthesized racemic mixture to get 100% (S)-nicotine.

b. In ECIG aerosols

The results obtained from the analysis of aerosols generated by ALVIN machine give an indication of the amount of nicotine entered into the body. Since e-liquids that originally contained (R) and (S)-nicotine still contained the same percentages after vaping on the filters, this means that interconversion from (S) to (R)-nicotine or from (R) to (S)-nicotine did not take place under the controlled experimental conditions. This is in agreement with the results reported in the literature, which indicate that interconversion does not take place at the vaping temperature (around 300^{o}C) [118].

3. Conclusion

Using the analytical method described above, we were able to quantify the concentration of nicotine and the percentages of nicotine enantiomers in several e-liquids and aerosols emitted from them. This quantification gave us a clue regarding the nature of nicotine present in the ECIGs, which is necessary to identify due to the regulations placed on tobacco-derived nicotine. In addition, it can be deduced that the form of nicotine, whether (R) or (S), that enters the body will be the same as that initially present in the e-liquid. Moreover, the presence of (R)-nicotine in the ECIG aerosol indicates that the behavior of nicotine absorbed from different e-liquids inside the body will not be the same, since the toxicities of nicotine enantiomers are different as mentioned in paragraph A.3.b.

In future work, pouches, which are being used for tobacco smoking cessation, will also be analyzed.

CHAPTER V

CONCLUSION

The results obtained from the systematic review and meta-analysis indicated that prenatal exposure to DDE is significantly associated with an increase in BMI z-score and overweight risk during childhood. These results highlight the potential role of persistent organic pollutants as endocrine disrupting chemicals that can lead to obesity. The negative impacts of these chemicals in addition to their presence in maternal serum and cord blood, call for the importance of implementing more strict regulations on the use and production of these chemicals. Moreover, it is important to keep monitoring the levels of these pollutants in humans by establishing more sensitive analytical instruments.

The results obtained from the analysis of nicotine enantiomers in e-liquids and aerosols emitted from ECIGs indicate that the form of nicotine, whether (R) or (S), that enters the body will be the same as that initially present in the e-liquid, without any (R)/(S) interconversion taking place during vaping. These results highlight the need for further studies on the toxicities of the nicotine enantiomers, with emphasis on the (R)-enantiomer due to the possibility of its use as a therapeutic agent.

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