Development of GC-MS/MS based affordable confirmatory analytical methodology for dioxins and PCBs in fish and fish products and exploration of a sustainable decontamination strategy

by

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This is to certify that the work incorporated in this Ph.D. thesis entitled, "**Development of GC-MS/MS based affordable confirmatory analytical methodology for dioxins and PCBs in fish and fish products and exploration of a sustainable decontamination strategy**" submitted by Kirankumar P.S. to the Academy of Scientific and Innovative Research (AcSIR) in partial fulfillment of the requirements for the award of the **Degree of Ph.D. in science**, embodies orginal research work carried out by my student.

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-Kirankumar P. S.

To my Amma, Achan, Sree & Kunjooz

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Abbreviations

PCBs	Poly Chlorinated Biphenyls
POPs	Persistent Organic Pollutants
PCDDs	Poly Chlorinated Dibenzo-p-Dioxins
PCDFs	Poly Chlorinated Dibenzo Furans
AhR	Aryl hydrocarbon Receptors
NO PCB	Non Ortho
MO PCB	Mono Ortho
NDL	Non Dioxin Like
PVC	Poly Vinyl Chloride
MSW	Municipal Solid Waste
BAN	Basel Action Network
ARNT	Aryl Nuclear Translocator
ROS	Reactive Oxidase Substance
TEQ	Toxic Equivalents
UB	Upper Bound
LB	Lower Bound
MB	Medium Bound
LOQ	Limit of Quantification
US-EPA	United States Environmental Protection Agency
EU	European Union
TDI	Tolerable Daily Intake
TWI	Tolerable Weekly Intake
TEF	Toxic Equivalents Factor
CPP	Citrus Pulp Pellets
ASE	Accelerated Solvent Extractor
PLE	Pressurised Liquid Extraction
SPME	Solid Phase Micro Extraction
SFE	Supercritical Fluid Extraction

MSPD	Matrix Solid Phase Dispersion
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
GPC	Gel Permeation Chromatography
CALUX	Chemically-activated luciferase expression
DRE	Dioxin Response Elements
GC- HR MS	Gas Chromatography High Resolution Mass spectrometery
GC- MS/MS	Gas Chromatography Tandem mass spectrometry
ML	Maximum Level
PBMS	Performance Base Monitoring System
IDMS	Isotope Dilution Mass Spectrometry
RRF	Relative Response Factor
ISTD	Internal Standard
RMS	Root Mean Square
LACP	Lowest Acceptable Calibration Point
MU	Measurement Uncertainty
dl	dioxin like
PBT/LRT	Persistent, Bio accululative Toxic Long Range Transport
DDT	dichloro-diphenyl-trichloroethane
CEPI	Comprehensive Environmental Pollution Index
WHO	World Health Organization
EFSA	European Food Safety Authority
TOC	Total Organic Carbon
BSAF	Biota Sediment Accumulation Factor
MRM	Multiple Reaction Monitoring
SEM	Scanning Electron Microscope
AC	Activated Carbon
BET	Brunauer–Emmett–Teller
BJH	Barrett-Joyner-Halenda
FAME	Fatty Acid Methyl Esters
FFA	Free Fatty Acids

AV	Acid Value
IUPAC	International Union for Pure and Applied Chemistry
PFO	Pseudo First Order
PSO	Pseudo Second Order
IPD	Intra Particle Diffusion
FT-IR	Fourier Transform Infrared Spectroscopy
PTFE	Polytetrafluoroethylene

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Introduction

1.1. Dioxins and PCBs – General overview

Dioxins and polychlorinated biphenyls (PCBs) are classes of persistent organic pollutants (POPs) regulated globally through the adoption and implementation of the Stockholm Convention, a global treaty on POPs (Stockholm Convention, 2004). They are highly toxic species that can trigger adverse health effects at ultra-trace levels (low picogram level). The term "dioxins" represents an umbrella term used to represent two classes of species, namely polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzo-p-furans (PCDFs). The chemical structure of PCDD/Fs (**Fig.1.1**) includes a framework of two benzene rings associated with two oxygen atoms (PCDDs) or a furan ring (PCDFs). The substitution of hydrogen atoms of the framework with chlorine atoms results in members of the each category, known as congeners. There are 75 compounds in the PCDDs family and 135 compounds in the PCDFs family, resulting in a total of 210 compounds.

The toxicity of these contaminants is directly proportional to their molecular planarity. In the framework, the presence of chlorine atoms in positions 2,3,7,8 results in the most planar/toxic congener, while subsequent substitutions result in less toxicity. In view of this, 7 PCDDs and 10 PCDFs congeners (total of 17 congeners) were identified to possess a common aromatic hydrocarbon receptor (AhR) mediated toxicity profile (Esser C.,2012). On the other hand, PCBs have the general structure of biphenyls, and substitutions of hydrogen with chlorine atoms lead to the formation of congeners. PCBs can be broadly classified into three major classes: non-ortho PCBs (NO PCBs), mono-ortho (MO) PCBs, and non-dioxin like (NDL) PCBs with four, eight and six congeners respectively. This classification is based on the presence or absence of chlorine atoms in the ortho positions (2,2’,6,6’). Considering the planarity and toxicity, NO and MO-PCBs are collectively called dioxin-like (dl) PCBs. Even though NDL-PCBs exhibit different mode of toxicity, their presence in a matrix shows the possibility of the formation of DL-PCBs and dioxins in the near future. Hence, they are also known as indicator PCBs. **Table 1.1** compiles individual congener name, abbreviation and their CAS registry number.

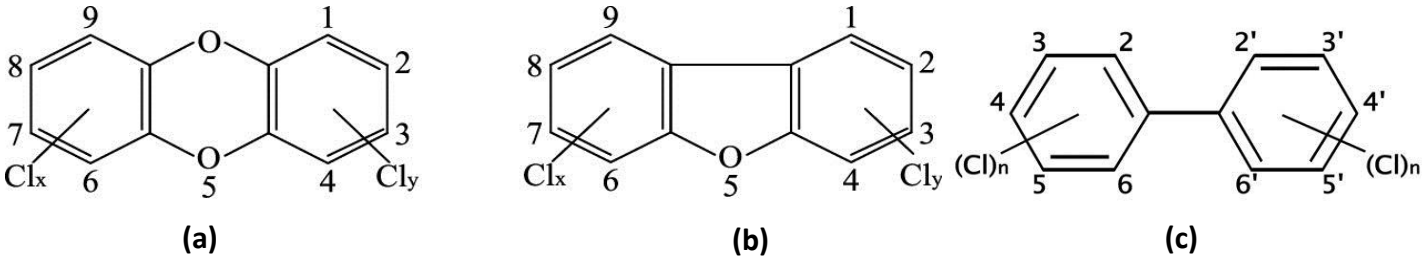


Fig.1.1 General structures of (a) PCDDs (b) PCDFs (c) PCBs

Table 1.1
List dioxins and PCBs under study

SI No	Compound class	Compound name	Abbrevation	CAS No
1	PCDDs	2,3,7,8-tetrachlorodibenzo-p-dioxin	2,3,7,8-TCDD	1746-01-6
2		1,2,3,7,8-pentachlorodibenzo-p-dioxin	1,2,3,7,8-PeCDD	40321-76-4
3		1,2,3,4,7,8-hexachlorodibenzo-p-dioxin	1,2,3,4,7,8-HxCDD	39227-28-6
4		1,2,3,6,7,8-hexachlorodibenzo-p-dioxin	1,2,3,6,7,8-HxCDD	57653-85-7
5		1,2,3,7,8,9-hexachlorodibenzo-p-dioxin	1,2,3,7,8,9-HxCDD	72918-21-9
6		1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin	1,2,3,4,6,7,8-HpCDD	35822-46-9
7		1,2,3,4,6,7,8,9-octachlorodibenzo-p-dioxin	1,2,3,4,6,7,8,9-OCDD	3268-87-9
8	PCDFs	2,3,7,8-tetrachlorodibenzofuran	2,3,7,8-TCDF	51207-31-9
9		1,2,3,7,8- pentachlorodibenzofuran	1,2,3,7,8-PeCDF	57117-41-6
10		2,3,4,7,8-pentachlorodibenzofuran	2,3,4,7,8-PeCDF	57117-41-6
11		1,2,3,7,8,9-hexachlorodibenzofuran	1,2,3,7,8,9-HxCDF	39227-32-2
12		1,2,3,4,7,8-hexachlorodibenzofuran	1,2,3,4,7,8-HxCDF	57117-31-4
13		1,2,3,6,7,8-hexachlorodibenzofuran	1,2,3,6,7,8-HxCDF	57653-86-8
14		1,2,3,7,8,9-hexachlorodibenzofuran	1,2,3,7,8,9-HxCDF	72918-22-0
15		1,2,3,4,6,7,8-heptachlorodibenzofuran	1,2,3,4,6,7,8-HpCDF	67562-41-8
16		1,2,3,4,7,8,9-heptachlorodibenzofuran	1,2,3,4,7,8,9-HpCDF	55673-89-7
17		1,2,3,4,6,7,8,9-octachlorodibenzofuran	1,2,3,4,6,7,8,9-OCDF	39001-02-0
18	NO-PCBs	3,3',4,4'-tetrachlorobiphenyl	PCB-77	32598-14-4
19		2,3,4,5,2',5'-hexachlorobiphenyl	PCB-81	53765-89-0
20		3,3',4,4',5-pentachlorobiphenyl	PCB-126	57465-28-8
21		3,3',4,4',5,5'-hexachlorobiphenyl	PCB-169	32774-16-6
22	MO-PCBs	2,2',4,4',5-pentachlorobiphenyl	PCB-123	25501-61-9
23		2,3',4,4',5-pentachlorobiphenyl	PCB-118	31508-00-6
24		2,3,3',4,4'-pentachlorobiphenyl	PCB-114	13874-02-7
25		2,3,3',4',5-pentachlorobiphenyl	PCB-105	32598-13-3
26		3,3',4,4',5,5'-hexachlorobiphenyl	PCB-167	52663-72-6

27		2,3,3',4,4',5-hexachlorobiphenyl	PCB-156	38380-08-4
28		2,3,4,4',5,5'-hexachlorobiphenyl	PCB-157	39635-31-9
29		2,3,3',4,4',5,5'-heptachlorobiphenyl	PCB-189	39227-28-6
30	NDL-PCBs	2,4,4'-trichlorobiphenyl	PCB-28	7012-37-5
31		2,2',5,5'-tetrachlorobiphenyl	PCB-52	35693-99-3
32		2,2',4,5,5'-pentachlorobiphenyl	PCB-101	31508-00-6
33		2,2',4,4',5,5'-hexachlorobiphenyl	PCB-153	35065-27-1
34		2,2',3,4,4',5-hexachlorobiphenyl	PCB-138	35065-28-2
35		2,2',3,4,4',5,5'-heptachlorobiphenyl	PCB-180	35065-29-3

Dioxins and PCBs exhibit high lipophilicity and hydrophobic behavior, as indicated by their octanol partition coefficient (log Kow) values ranging from 6 to 12 (Mahfouz et al., 2020). This property raises concerns because a significant portion of the human diet comprises fat-rich foods such as meat, fish, poultry, milk, and their products. Consequently, ingestion is considered the major route of exposure to humans (>90 %) (Weir., 2005). In addition to their lipophilicity, another critical parameter is their extended half-life, particularly notable in species like 2,3,7,8-TCDD, which ranges from 7 to 11 years in human beings (Milbrath et al., 2009). This prolonged half-life facilitates transport of these contaminants through food chains, thereby posing risks to the entire food web. Initially, dioxins and PCBs accumulate in environmental compartments such as sediment and soil before entering the food chain. Regulatory bodies are therefore vigilant about monitoring these contaminants in both environmental and food matrices. They establish analytical methodologies and set maximum permissible levels to mitigate potential risks associated with exposure to these substances.

Dioxins, primarily unintentionally produced compounds, saw their toxic effects recognized in the 20th century (during world war II), leading to heightened awareness of their environmental and health impacts. Meanwhile, PCBs found extensive use in electrical equipment, such as transformers, capacitors, and heat exchange devices (Erickson & Kaley., 2011). They were manufactured worldwide until their ban in numerous countries due to potential environmental and health concerns. Seveso disaster (1976), Love Canal Contamination (1978), Times Beach Contamination (1982**),** Dioxin Contamination in Vietnam- agent orange issue (1960s-1970s) etc are the major outbreak incidents that prompted widespread surveillance and spurred research

and development efforts aimed at understanding and addressing these contaminants (Eskenazi et al., 2018).

1.2. Sources, formation pathways and health effects

1.2.1. Major dioxin/PCBs sources

Dioxins and PCBs pervade our environment, stemming from a myriad of sources. The presence of chlorine, organic matter, and specific temperature conditions fosters their formation**. Fig 1.2** illustrate various significant sources of these contaminants. In contemporary waste management practices, incineration stands out as a pivotal tool globally, revered for its ability to drastically reduce waste volume and minimize environmental impact. However, the combustion process inadvertently generates dioxins, especially when incinerating chlorine-rich materials like PVC plastics or chlorinated organic compounds.

The intense heat and intricate chemical reactions during combustion lead to the restructuring of chemical bonds, giving rise to dioxin formation. Notably, biomedical waste incinerators emerge as significant dioxin emitters due to the prevalence of polyvinyl chloride (PVC) based materials in medical waste (Zhang et al., 2015). Developing economies heavily rely on biomedical waste incineration to manage their waste load, thereby elevating these incinerators to the forefront of dioxin emissions inventories in such nations (Ajay & Prathish., 2024). Despite the potential of modern biomedical waste incinerators, equipped with advanced pollution control technologies, to effectively mitigate dioxin emissions to levels well below regulatory thresholds, their widespread adoption is hindered by lack of awareness regarding the risks associated with these contaminants.

Municipal solid waste (MSW) incineration plays a significant role in waste management strategies worldwide, offering a solution for the disposal of non-recyclable and non-compostable waste (Wei et al., 2022). However, it comes with environmental concerns, particularly regarding the emission of dioxins. Dioxins are unintentionally produced during incomplete combustion processes, with MSW incineration being a notable source due to the complex composition of municipal waste. Studies have shown that dioxin emissions from MSW incinerators can vary widely depending on factors such as waste composition, incinerator technology, and operational practices. For example, according to data from the U.S. Environmental Protection Agency (EPA), MSW incinerators are estimated to contribute approximately 5% of total dioxin

emissions in the United States (EPA, 2017). Despite advancements in pollution control technologies, such as baghouse filters and scrubbers, which can significantly reduce dioxin emissions from MSW incinerators, the potential for dioxin formation remains a concern. Therefore, efforts to improve waste segregation, increase recycling and composting, and promote alternative waste treatment methods are essential for minimizing the environmental impact.

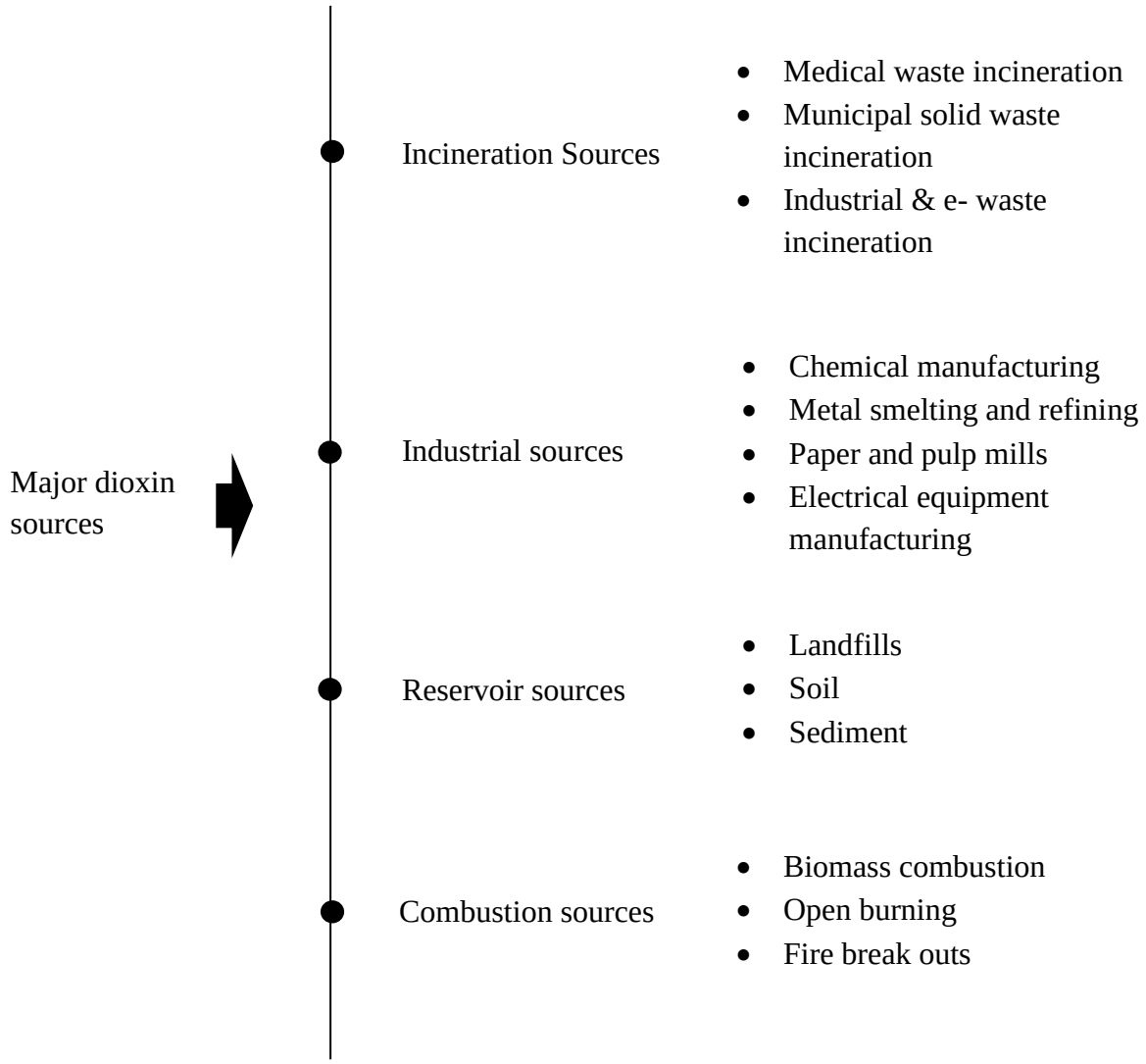


Fig. 1.2 Major sources of dioxins and PCBs

E-waste incineration poses a unique challenge in waste management due to the diverse array of materials it contains, including various toxic substances. Of particular concern are dioxins, which are prominently present in e-waste due to the prevalence of chlorinated materials like PVC plastics and flame retardants commonly, found in electronic devices (Tue et al., 2013). Research, such as that conducted by the Basel Action Network (BAN), has shown that e-waste incinerators in developing countries emit dioxins at significantly elevated levels compared to those in industrialized nations (BAN). In many cases, these emissions surpass regulatory limits by several orders of magnitude, highlighting the pressing need for effective management strategies to mitigate the environmental and health risks associated with e-waste incineration.

Industrial activities such as chemical manufacturing, metal smelting and refining, paper and pulp mills, and electrical equipment manufacturing are significant sources of dioxin emissions (Ritchie., 2000). These sectors often involve processes that utilize or produce chlorine-containing compounds, contributing to the release of dioxins into the environment. For example, chemical manufacturing processes can generate dioxins as byproducts during the production of certain chemicals, with studies indicating that these facilities can be significant contributors to dioxin emissions. Similarly, metal smelting and refining operations, particularly those involving the recycling of electronic scrap or the processing of chlorinated materials; have been identified as sources of dioxin contamination. In paper and pulp mills, chlorine bleaching processes used to whiten paper products can lead to the formation of dioxins as unintended byproducts (Thacker et al., 2007). Additionally, electrical equipment manufacturing, which often involves the use of PVC plastics and other chlorinated materials, can result in dioxin emissions during production processes. According to data from regulatory agencies and environmental organizations, industrial sources collectively account for a substantial portion of dioxin emissions globally, underscoring the importance of implementing stringent regulations and pollution control measures in these sectors to minimize environmental and public health risks.

Reservoir sources such as landfills, soil, and sediment play a significant role in the distribution and persistence of dioxins in the environment. Landfills serve as repositories for various types of waste, including industrial, municipal, and hazardous materials, many of which contain dioxin-producing substances such as PVC plastics and chlorinated organic compounds. Over time, these materials can undergo decomposition and chemical reactions, leading to the release of dioxins

into the surrounding soil and groundwater (Kulkarni et al., 2007). Similarly, soil acts as a reservoir for dioxins due to the deposition of airborne contaminants, as well as the application of sewage sludge or contaminated fertilizers. Dioxins can persist in soil for extended periods, posing risks to ecosystems and human health through direct contact or uptake by plants (Tran et al., 2022). Sediment in aquatic environments also serves as a sink for dioxins, accumulating contaminants from upstream sources such as industrial discharges or runoff from contaminated land. Once deposited in sediment, dioxins can become trapped and persist for long periods, potentially impacting aquatic organisms and bio accumulating in the food chain (Beaubien et al., 2023). Effective management of these reservoir sources is essential for preventing further contamination and reducing the risk of exposure to dioxins in the environment.

Combustion sources, including biomass combustion, open burning, and fire breakouts, are significant contributors to dioxin emissions in the environment (Kumari et al., 2019; Ajay et al., 2022). Biomass combustion, such as burning wood or agricultural residues for heating or energy generation, can release dioxins when organic materials containing chlorine, such as treated wood or agricultural pesticides, are burned incompletely. Open burning, which involves the uncontrolled combustion of waste materials in outdoor settings, is a common practice for waste disposal in many regions. However, open burning of waste, including household trash, plastics, and electronic waste, can produce dioxins as byproducts due to incomplete combustion and the presence of chlorine-containing materials. Fire breakouts, such as wildfires or accidental fires in industrial facilities or residential areas, can also generate dioxins when burning materials containing chlorinated compounds, such as building materials, plastics, or electrical equipment (Vassiliadou et al., 2008). These combustion sources release dioxins into the atmosphere, where they can be transported over long distances and deposited onto land or water surfaces, posing risks to human health and the environment. Effective measures to control combustion sources, such as implementing regulations to reduce open burning and wildfires and promoting cleaner combustion technologies, are essential for minimizing dioxin emissions and protecting public health and ecosystems.

1.2.2. Formation pathways

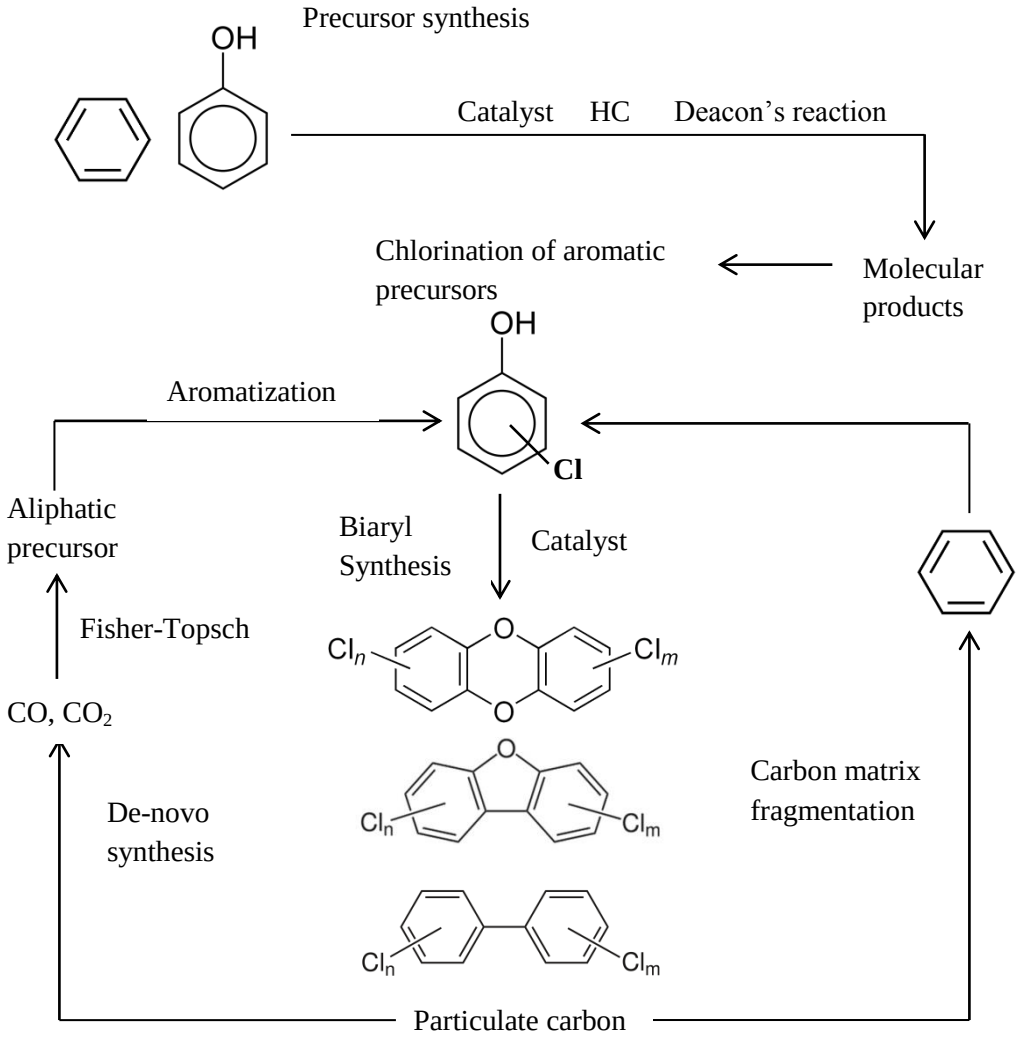


Fig. 1.3. Formation pathways of dioxins and PCCBs through precursor pathway and de novo synthesis

Two major reaction mechanisms are proposed for the formation of dioxins and PCBs: de novo synthesis and the precursor pathway (Wang et al., 2022). De novo synthesis involves the formation of dioxins from particulate carbon and chlorine sources, typically occurring at elevated temperatures during combustion processes. Initially, particulate carbon is converted to CO and CO_2. The resulting CO and H_2 (syngas) undergo the Fisher-Tropsch process to form aliphatic precursors. These precursors undergo aromatization in the presence of chlorine to yield

chlorinated aromatic species, which further give rise to dioxins and PCBs. In contrast, the precursor route involves the formation of dioxins and PCBs from similar aromatic compounds with chlorine (e.g., chlorinated pesticides) or from aromatic compounds like phenol/benzene, where chlorine is supplied from HCl through Deacon's process (Zhou & Liu., 2018). This pathway is significant in waste incineration, industrial processes involving chlorine-containing materials, and other combustion activities where precursor compounds are present. Studies have shown that by analyzing the fingerprint of dioxin congeners, it is possible to determine which mechanism contributes more to the formation of dioxins. The de novo mechanism primarily results in the formation of polychlorinated dibenzofurans (PCDFs), while the precursor pathway tends to favor the production of polychlorinated dibenzo-p-dioxins (PCDDs). Analyzing the ratio between ∑PCDD/∑PCDF can confirm the formation mechanism (∑PCDD/∑PCDF > 1 indicates the precursor route, while ∑PCDD/∑PCDF < 1 indicates de novo synthesis) (Huang and Buekens, 1995). **Fig.1.3** represents a schematic representation of dioxins/PCBs formation through the precursor and de novo synthesis pathways

1.2.3. Health effects

Fig 1.4 depicts the overview of health issues pertaining to dioxins/PCBs. Exposure to dioxins and PCBs in humans leads to a range of significant health issues. One prominent effect is the development of a skin disorder known as chloracne, characterized by acne-like lesions and cysts primarily on the face and neck. Chloracne serves as a hallmark sign of dioxin/PCB poisoning. Additionally, dioxins and PCBs contribute to heart and circulatory problems by disrupting normal cellular functions, leading to oxidative stress, inflammation, and damage to blood vessels. This disruption can contribute to the development of cardiovascular diseases such as atherosclerosis, hypertension, and heart disease (White & Birnbaum., 2009)

Dioxin exposure has also been linked to changes in lipid metabolism and blood clotting factors, further increasing the risk of heart and circulatory issues. Chronic exposure to these contaminants is associated with various liver-related health problems, including hepatitis, fatty liver and liver cancer. In males, dioxin exposure leads to decreased sperm quality and quantity, as well as abnormalities in sperm morphology and motility. In females, it results in menstrual irregularities and reduced ovarian function, ultimately leading to reduced reproductive capacity.

Furthermore, dioxins are implicated in triggering various types of cancers and suppressing the immune system. Given the broad spectrum of health effects associated with dioxins and PCBs, widespread surveillance of these contaminants is necessary to ensure human safety.

Dioxins and PCBs typically bind with aryl hydrocarbon receptors (AhR) within cells, leading to adverse health effects. In a review by Furue et al., the association between AhR and dioxin-related health hazards is examined, detailing the mechanism of dioxin toxicity pathway (Furue et al., 2021) (**Fig. 1.5**). Initially, AhR in the cytoplasm is bound to proteins like HSP90-XAP-2-P23. Upon interaction with dioxins, AhR releases these proteins and binds with AhR nuclear translocator (ARNT). The resulting dioxin-AhR-ARNT complex then binds with the xenobiotic response elements (XREs) of target genes in the nucleus. This binding ultimately leads to the production of reactive oxygen species (ROS), which contribute to the adverse health effects of dioxins and PCBs.

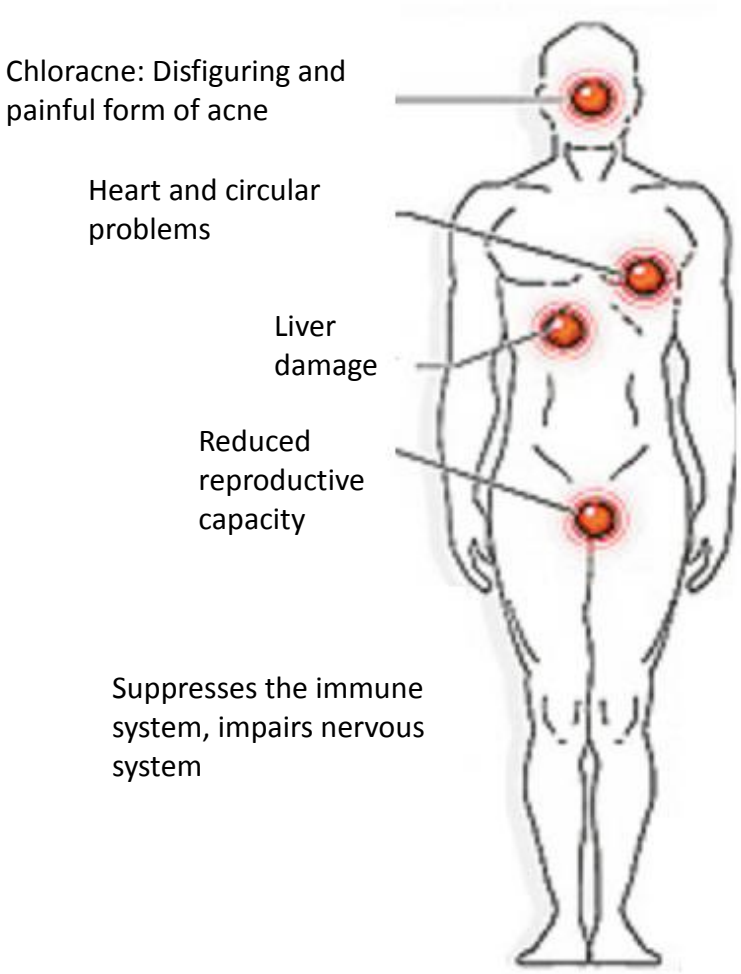


Fig. 1.4 Heath effects of dioxins and PCBs (Dioxins - Health Effects – Infographic, 2011)

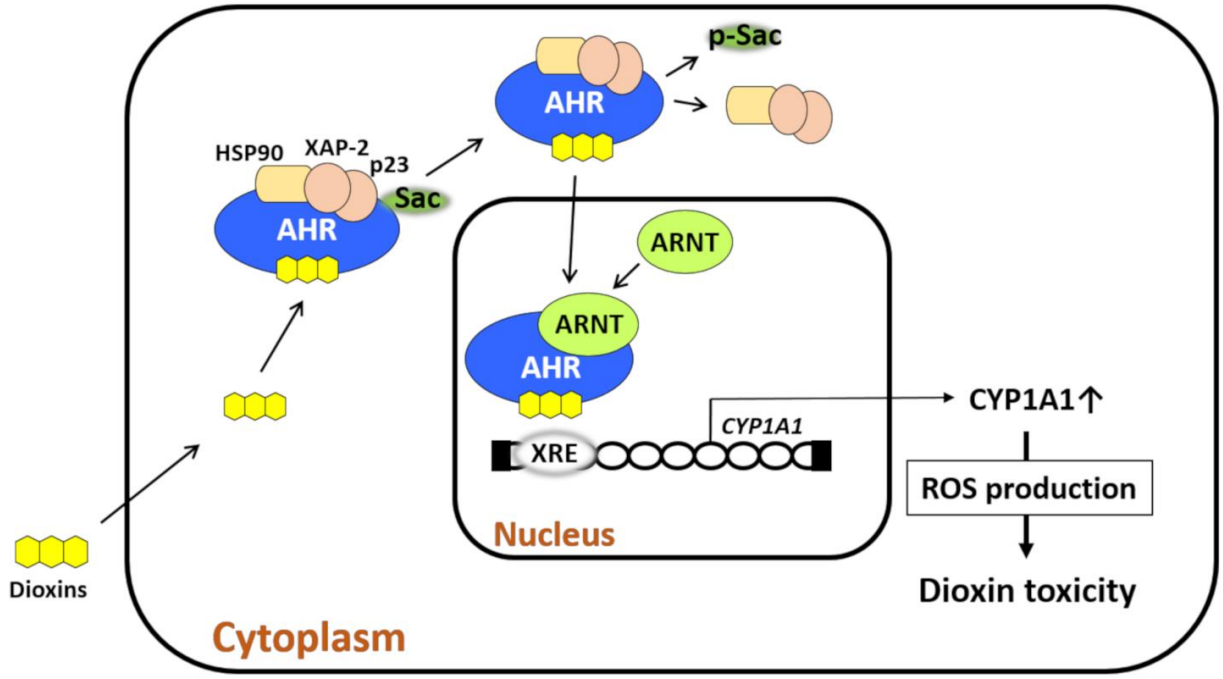


Fig. 1.5 Toxicity pathways of dioxins/PCBs pathway (Furue et al., 2021)

1.3. Environmental fate, persistence and bioaccumulation

Dioxins and PCBs, characterized by their persistent, bio accumulative, toxic, and long-range transport properties, pose significant environmental challenges. Their remarkable stability makes them resistant to degradation processes such as microbial activity, sunlight, and chemical reactions. Consequently, once released into the environment, these compounds tend to persist for extended periods, accumulating in various environmental compartments like soil, sediment, and fly ash. Their stability is attributed to their molecular structure, which lacks reactive sites vulnerable to degradation. Additionally, their high lipophilicity contributes to their tendency to accumulate in the fatty tissues of organisms. The environmental fate of dioxins and PCBs involves a complex interplay of processes. Once released, they adhere to surfaces and are gradually transferred through environmental compartments (Rappe et al., 1987). This persistence in the environment facilitates their bioaccumulation, a process by which these compounds accumulate in living organisms over time. As lower trophic level organisms ingest contaminated food or water, dioxins and PCBs accumulate in their tissues. Subsequently, when these

organisms are consumed by higher trophic level organisms, the accumulated contaminants are transferred, leading to bio magnification. This phenomenon results in higher concentrations of dioxins and PCBs in organisms higher up the food chain. The long-range transport of dioxins and PCBs further increases their environmental impact. These compounds can travel over considerable distances through air and water currents, leading to their widespread distribution beyond their original source (Lohman & Seigneur., 2001). Consequently, regions far removed from industrial or agricultural activities can still be affected by these contaminants. The combination of persistence, bioaccumulation, and long-range transport underscores the importance of addressing dioxins and PCBs as significant environmental pollutants. Efforts to mitigate their adverse effects require comprehensive strategies aimed at reducing their release into the environment and minimizing their bioaccumulation and bio magnification in ecosystems.

1.4. Concept of TEQ and reporting

To quantify the toxicity of dioxins/PCBs across different matrices, a concept called toxicity equivalence (TEQ) is utilized, providing a measure of the cumulative effect of monitored congeners. Each congener is assigned a toxic equivalence factor (TEF) based on its toxicity relative to the most toxic congener, 2,3,7,8-TCDD (Berg et al., 2006). For instance, the TEF value assigned to 1,2,3,4,7,8-HxCDD is 0.1, indicating its toxicity is one-tenth that of 2,3,7,8-TCDD. The cumulative effect of congeners is determined using **Eq 1.1**, where Ci represents the individual concentration of the congener. Once the TEQ value is calculated, it needs to be expressed in units corresponding to the matrix being analyzed. For example, the maximum permissible level of dioxins in fish and feed-grade fish oil is expressed in pg TEQ/g wet weight and pg TEQ/g relative to a feed with 12% moisture content respectively. In the case of non-dioxin-like PCBs (NDL-PCBs), as they exhibit lower toxicity, they are not assigned TEF values, and the cumulative effect is expressed as the sum of individual concentrations. **Table 1.2** presents the WHO-assigned TEF values as of 1998 alongside their corresponding updates in 2005.

$$TEQ = \sum_{i=1}^{17}(C_{i(PCDD/Fs)} * TEF_i) + \sum_{i=1}^{12}(C_{i(dl-PCBs)} * TEF_i) \text{---------(Eq 1.1)}$$

The upper bound (UB), medium bound (MB), and lower bound (LB) concept is utilized to represent the levels of dioxins/PCBs. The congener-based limit of quantification (LOQ) must be

established for the analytical protocol used to quantify dioxins and PCBs. The LOQ denotes the minimum quantity of analyte that the analytical protocol can quantify with statistical certainty. The following methodology is employed to calculate UB, MB, and LB, and this concept is particularly significant when the analyte concentration is lower than the LOQ. It results in a range where the analyte concentration can vary, providing more certainty.

$$If\ analyte\ cooncntration > LOQ => UB = MB = LB$$

$$If\ analyte\ cooncntration < LOQ => UB = LOQ, MB = \frac{LOQ}{2}, LB = 0$$

Table 1.2
WHO-dioxins and dioxin like PCBs TEF values

SI No	Category	Compounds	TEF-1998	TEF-2005
1	PCDDs	2,3,7,8- TCDD	1	1
2		1,2,3,7,8- PeCDD	1	1
3		1,2,3,4,7,8- HxCDD	0.1	0.1
4		1,2,3,6,7,8- HxCDD	0.1	0.1
5		1,2,3,7,8,9- HxCDD	0.1	0.1
6		1,2,3,7,8,9- HpCDD	0.01	0.01
7		OCDD	**0.0001**	**0.0003**
8	PCDFs	2,3,7,8-TCDF	0.1	0.1
9		1,2,3,7,8-PeCDF	**0.05**	**0.03**
10		2,3,4,7,8-PeCDF	**0.5**	**0.3**
11		1,2,3,4,7,8-HxCDF	0.1	0.1
12		1,2,3,6,7,8- HxCDF	0.1	0.1
13		1,2,3,7,8,9- HxCDF	0.1	0.1
14		2,3,4,6,7,8- HxCDF	0.1	0.1
15		1,2,3,4,6,7,8- HpCDF	0.01	0.01
16		1,2,3,4,7,8,9- HpCDF	0.01	0.01
17		OCDF	**0.0001**	**0.0003**
18	NO-PCBs	PCB-77	0.0001	0.0001
19		PCB-81	**0.0001**	**0.0003**
20		PCB-126	0.1	0.1
21		PCB-169	**0.01**	**0.03**
22	MO-PCBs	PCB-105	0.0001	0.00003

23	PCB-114	0.0005	0.00003
24	PCB-118	0.0001	0.00003
25	PCB-123	0.0001	0.00003
26	PCB-156	0.0005	0.00003
27	PCB-157	0.0005	0.00003
28	PCB-167	0.00001	0.00003
29	PCB-189	0.0001	0.00003

1.5. Regulatory aspects

Regulatory bodies worldwide have implemented stringent regulations and analytical methods to quantify dioxins and PCBs, due to their toxicity and adverse health effects. These regulations typically cover three critical matrices: environmental (such as soil and sediment), food, and feed. The primary regulatory bodies overseeing these efforts are the Environmental Protection Agency (US-EPA) and the European Union (EU). While the US-EPA has primarily focused on environmental matrices, the EU has concentrated on food and feed matrices. **Table 1.3** and **1.4** outlines the regulations and analytical methods established by these regulatory bodies over time. Regardless of the matrix type, regulatory bodies have set action levels and maximum levels for these contaminants. The action level serves as the threshold concentration in a particular matrix. Exceeding this level triggers further investigation, enforcement measures, or the implementation of pollution control measures to reduce contaminant presence. On the other hand, the maximum level represents the highest allowable concentration permitted by regulatory standards. Levels exceeding this threshold are associated with significant health risks. In summary, **Table 1.5** provides a summary of the assigned maximum and action levels established for selected matrices by different regulatory bodies. These thresholds are essential for safeguarding public health and the environment from the harmful effects of dioxins and PCBs. International organizations such as the WHO and EFSA have set Tolerable Daily Intake (TDI) values for dioxins/PCBs. The WHO established a TDI range of 1 - 4 TEQ pg/kg bw, while EFSA recently introduced a more stringent Tolerable Weekly Intake (TWI) value equivalent to 2 pg TEQ/kg bw/week (Van Leeuwen et al., 2000; EFSA CONTAM Panel, 2018).

Table 1.3

US-EPA analytical methods/ tools for quantifying dioxins and PCBs in various matrices

SI No	Analytical method/tools	Highlights	Reference
1	EPA Tool kit	Crafted to aid nations in establishing release inventories of polychlorinated dibenzo-p-dioxins and dibenzofurans on a national or regional scale.	(EPA Toolkit , 2013)
2	EPA-Database	Comprising a comprehensive array of databases related to dioxins, encompassing inventories, risk assessments, pertinent surveys, and more.	(EPA Dioxin database)
3	Method 23/23A	This method is designed to quantify air emissions of dioxins and furans originating from stationary point sources. It outlines the sampling strategy and details the calculation procedures for determining overall emission results.	(EPA Method 23 A, 2018)
4	Method 4430	This method screens soil and sediment samples specifically for total polychlorinated PCDDs and PCDFs), excluding polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). It employs the aryl hydrocarbon receptor (AhR)-based polymerase chain reaction (PCR) assay to estimate the overall toxicity of the total PCDD/F mixture detected.	(EPA Method 4425 , 2007)
5	Method 1613B	This method quantifies tetra- through octa-chlorinated polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) in environmental matrices using high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). Isotope dilution is employed to accurately quantify very low concentrations of individual PCDD and PCDF congeners.	(Method 1613 B, 1994)
6	Method TO-9A	This procedure outlines the collection, preparation, and analysis of polyhalogenated dioxins, encompassing polychlorinated, polybrominated, and	(Method TO-9 A, 1999)

		brominated/chlorinated dibenzo-p-dioxins and dibenzofurans present in ambient air.	
7	Method 8280B	This method measures the concentrations of individual 2,3,7,8-substituted tetra- through octa-chlorinated PCDDs and TCDFs, albeit with less sensitivity compared to Method 1613B. Utilizing HRGC/low resolution MS, it is capable of quantifying individual dioxins in fly soil and ash at levels ranging from 1 to 5 parts per million (ppm). Additionally, it enables the quantification of individual dioxins in water, fuel oil, oily sludges, or other chemical waste samples at concentrations ranging from 10 to 50 parts per trillion (ppt).	(EPA Method 8280, 2007)
8	Method 8290A	This method employs HRGC/high resolution MS to measure tetra- through octa-chlorinated PCDDs and TCDFs in various matrices, including soil, sediment, fly ash, water, sludge (including paper pulp), still bottom, fuel oil, chemical reactor residue, fish tissue, or human adipose tissue. The higher resolution MS, compared to Method 8280B, enables the detection and quantification of these compounds at concentrations ranging from parts-per-trillion (ppt) to parts-per-quadrillion (ppq).	(EPA Method 8290 A, 2007)

Table 1.4

EU regulations significant to dioxins and PCBs

SI No	Regulation	Highlights	Reference
1	Commission Regulation (EC) No 1881/200	This regulation establishes maximum permissible levels for specific contaminants in food products.	(Commission Regulation, 2011)
2	Commission Regulation (EU) 2017/644	This regulation specifies methods for sampling and analyzing to monitor the levels of dioxins, dioxin-like PCBs, and non-dioxin-like PCBs in certain food products.	(European Union, 2017)
3	Commission Recommendation 2013/711/EU	Regarding the reduction of dioxins, furans, and PCBs in feed and food, as amended by Commission Recommendation 2014/663/EU.	(Commission regulation, 2013)

Table 1.5

Assigned maximum/ action levels in selected matrices

SI No	Regulatory body	Matrix	Maximum/ Action level	Reference
1	Canada	Sediment	21.5 pg TEQ/g (dioxin)	(Suzuki et al., 2016)
2	Japan		150 pg TEQ/g (dioxin)	
3	Netherlands		1000 pg TEQ/g (dioxin)	
4	US-EPA	Soil	<240 ng/kg (dioxin)	(USEPA, 2010)
5	EU	Fish	3,5(ML), 1.50 (AL) pg TEQ/g wet weight (PCDD/Fs) 6,5(ML), 2.50 (AL)TEQ/g wet weight (PCDD/Fs + dl-PCBs) 125 ng/g wet weight (ML) (ndl-PCBs)	(Commission Regulation, 2011)
6		Egg	1.75(ML,AL) pg TEQ/g fat (PCDD/Fs) 3 (ML), 1.75 (AL)TEQ/g fatt (PCDD/Fs + dl-PCBs) 40 ng/g fat (ML) (ndl-PCBs)	
7		Milk	2 (ML), 1.75 (AL) pg TEQ/g fat (PCDD/Fs) 4(ML), 2.0 (AL)TEQ/g fat (PCDD/Fs + dl-PCBs) 40 ng/g fat (ndl-PCBs)3,5 pg TEQ/g fat (PCDD/Fs)	
8		Fish oil (feed)	5 (ML), 4 (AL) pg TEQ/g (PCDD/Fs) 20 (ML), 11 (AL)TEQ/g (PCDD/Fs + dl-PCBs) 175 ng/g (ML) (ndl-PCBs) (weight is relative to a feed with 12% moisture content)	(European Union, 2009)

1.6. Dioxins/PCBs outbreaks/crisis related to food and feed

a) Citrus pulp case (A global concern)

In 1997, a significant environmental and economic crisis unfolded in Germany and the Netherlands, known as the Citrus Pulp Dioxin Crisis. The issue surfaced when milk and butter samples from these countries exhibited elevated levels of dioxins, specifically 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD. A thorough investigation revealed that the contamination stemmed from citrus pulp pellets (CPP) used in cattle feed. The primary source of contamination was traced back to the use of lime in the drying process of CPP, a substance originating from an area near a landfill site belonging to a chemical company which manufactures PVC. This revelation not only pointed to a serious breach in food safety but also had severe economic repercussions in many countries in Europe and South America (Malisch et al., 2000).

b) Belgian dioxin crisis

In 1999, Belgium faced a significant crisis when poultry farms across the country were found to be contaminated with dioxins. The source of this contamination was traced back to the feed used in these farms, which, in turn, was tainted by dioxins from animal fat used in its manufacture. The major component identified in the contamination was polychlorinated biphenyl (PCB). The discovery of these toxic substances in the food supply raised serious concerns about public health and prompted swift action from authorities. In response to the crisis, regulations were implemented to ensure the safety of animal feed, preventing a recurrence of such contamination. However, the economic consequences of the Belgium Dioxin Crisis were substantial, with estimates suggesting losses amounting to a staggering one billion euros. This crisis underscored the importance of stringent regulations and vigilant oversight to safeguard both public health and the economic stability of the agricultural sector (Barone et al., 2021).

c) Guar gum India

In 2007, India faced a significant crisis related to guar gum, a versatile substance widely used as a thickening agent and binder in various food products. The issue came to light when high levels of dioxins and pentachlorophenol were identified in guar gum exports from India to Switzerland. As a response, all consignments of guar gum from India were scrutinized, revealing elevated

levels of contaminants in specific shipments. Further investigation indicated that while the inherent levels of contaminants in guar gum were low, the issue arose during the processing of the substance. An inspection mission determined that the source of contamination was penta chlorophenate, employed in the production process of industrial-grade guar gum, leading to cross-contamination with the food-grade variant. Representatives from the European Union who visited India during this crisis observed a lack of control measures, emphasizing that basic surveillance of such contaminants was in its infancy stages in the country. The guar gum crisis underscored the importance of stringent production practices and robust surveillance systems in developing countries (EFS 2017)

d) Clay contamination

In 1994, a significant incident unfolded in Mississippi, shedding light on the alarming contamination of dioxins in farmed catfish. Investigations revealed elevated levels of dioxins in the fish, with a particular focus on the fish meal component, soybean, as a major source of contamination. Subsequent studies traced the issue back to specific clay employed as a flowing agent during soybean production. It was determined that this clay, crucial to the manufacturing process, was heavily contaminated with dioxins and PCBs. Through meticulous analysis of congener patterns, scientists were able to ascertain that the contamination of the clay was a result of the geothermal process employed. This revelation underscored the intricate interplay between industrial processes and environmental impact, emphasizing the need for rigorous quality control measures to safeguard the integrity of our food supply and ecological systems (Franzblau et al., 2008).

e) Buffalo milk contamination (Mozzarella Incident)

In the 2007-2008 period, a concerning incident known as the Mozzarella Incident unfolded in Italy, revealing alarming levels of dioxin and PCB contamination in mozzarella samples derived from buffalo milk. The investigation led to the identification of the contamination source—illegal deposition of toxic waste in proximity to buffalo farms, both domestically and globally. The significant quantities of contaminated cheese exported from Italy underscored the widespread nature of the risk, extending beyond specific regions. In response to this crisis, Italy conducted an extensive surveillance study employing confirmatory analytical protocols. This

incident served as a stark reminder of the potential for unforeseen contamination events, necessitating immediate surveillance studies. The global implications highlighted the urgency of developing robust analytical protocols, especially developing/ economies in transition, to proactively safeguard food safety and environmental health in the face of emerging risks (Cavallo et at., 2021)

f) Ireland pork contamination

The Ireland pork dioxin contamination incident occurred in December 2008 when Irish authorities discovered that pork and pork products produced in Ireland were contaminated with dioxins. Dioxins are toxic chemicals that can have adverse effects on human health, including potential links to cancer. The contamination was traced back to contaminated animal feed supplied to pig farms. The contaminated feed was found to contain high levels of dioxins, and it was determined that the source of the contamination was contaminated oil used in the manufacturing of the feed. This contaminated oil had been distributed to numerous pig farms across Ireland. As a precautionary measure, the Irish government issued a recall of all pork and pork products produced from the affected farms, leading to a temporary ban on Irish pork exports. The incident prompted a thorough investigation into the cause of the contamination, and measures were taken to address the issue and prevent its recurrence. It highlighted the importance of stringent monitoring systems in the food production chain and the potential for environmental contaminants to enter the food supply. The Irish government, in collaboration with relevant agencies, undertook corrective actions to ensure the safety of the food supply chain and to rebuild confidence in Irish pork products both domestically and internationally (Marnane et al., 2012)

1.7. Analytical techniques in monitoring of dioxins/PCBs

1.7.1. Sample extraction

Analytical methodologies such as solid-liquid extraction for solid sample matrices and liquid-liquid extraction for liquid sample matrices are generally employed for dioxins and PCBs. The Soxhlet extraction technique is the classical methodology specified by various international regulatory bodies as the standard for conducting solid sample extraction. However, drawbacks include prolonged sample extraction times, such as the 16-hour Soxhlet extraction for dioxins

and PCBs in fish tissue samples. Efforts are underway to mitigate this drawback by incorporating parallel lines, i.e., integrated systems with more than one Soxhlet flask. Considering these drawbacks, the scientific community is in constant pursuit of developing cost-effective analytical methodologies. Recent advances in this analytical procedure are outlined in the systematic review below.

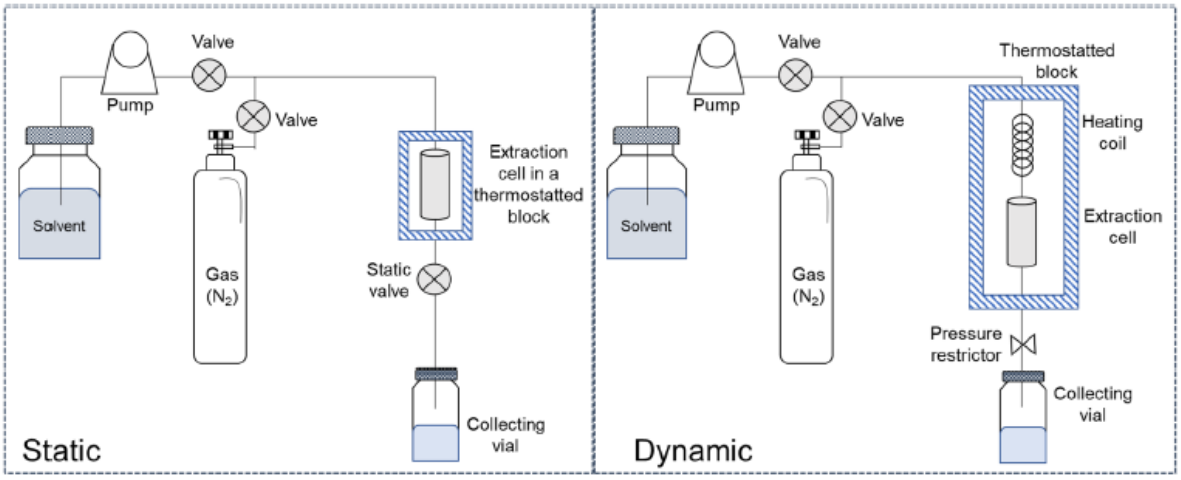


Fig.1.6 Schematic representation of Pressurized Liquid Extraction (PLE) system working in static and dynamic mode (Barp et al., 2023)

Accelerated Solvent Extraction (ASE) has become a pivotal technique in the field of dioxin analysis, providing a rapid and efficient means of extracting persistent organic pollutants from complex sample matrices. In contrast to traditional extraction methods, ASE employs elevated temperatures and pressures to enhance the efficiency of the extraction process. This automated technique significantly reduces extraction times while maintaining a high level of precision. In the context of dioxin analysis, where trace levels are of utmost importance, ASE ensures thorough extraction of analytes from various sample types, such as soils, sediments, and biological tissues. **Fig.1.6** gives schematic representation of PLE. Nording et al used accelerated solvent extraction methodology to analyze dioxin and PCBs content in food and feed matrices and observed similar performance as that of classical Soxhlet based sample extraction (Nording et al., 2006). The high throughput and reduced solvent consumption associated with ASE make it particularly advantageous for large-scale environmental monitoring studies. Moreover, its automated nature minimizes the risk of human error, ensuring reproducibility and reliability in dioxin analyses cleanup process not only simplifies the analytical workflow but also minimizes

the risk of analyte loss or degradation. In the realm of dioxin analysis, innovative techniques such as Solid Phase Micro Extraction (SPME), Supercritical Fluid Extraction (SFE), QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), Matrix Solid-Phase Dispersion (MSPD), and advanced sorbent and materials-based extraction have revolutionized sample preparation and analytical methodologies (Tu & Chen., 2018; Nieves et al., 2017). SPME's direct extraction approach using chemically coated fibers eliminates extensive solvent usage and laborious preparation steps, offering selective extraction of dioxins from complex matrices. SFE's use of supercritical fluids efficiently extracts dioxins while reducing sample processing time and minimizing analyte degradation risks. QuEChERS' single-step extraction and cleanup method streamline sample preparation, making it cost-effective and rapid for large-scale monitoring studies. MSPD integrates extraction, cleanup, and concentration into a single process, ensuring accurate dioxin analysis in diverse samples. Moreover, advancements in sorbents and materials enhance selectivity and efficiency while contributing to greener extraction practices. These techniques collectively represent significant advancements in dioxin analysis, providing researchers with robust, efficient, and environmentally sustainable tools for environmental/food monitoring and public health assessment.

1.7.2. Sample clean-up

a) **Adsorption chromatography**

Adsorption chromatography, utilizing multilayer silica, alumina, and carbon columns, emerges as a powerful and versatile technique in the realm of dioxin analysis. This innovative approach showcases the synergy of three distinct materials, each contributing unique properties to enhance separation and detection capabilities. Silica, known for its surface interactions and selectivity, teams up with alumina and carbon to form a robust multilayer matrix that facilitates the effective separation of dioxins. The application of this chromatographic method significantly improves the resolution and sensitivity of dioxin analysis, allowing for a more comprehensive understanding of complex environmental samples. The choice of multiple layers offers a dynamic platform for tailoring separation conditions, enabling scientists to fine-tune parameters for specific dioxin congeners. This approach not only enhances the efficiency of chromatographic separation but also showcases the versatility of multilayer systems in tackling challenging analytical tasks. As the demand for precise and reliable dioxin analysis continues to

grow, the integration of multilayer silica, alumina, and carbon columns proves to be a cutting-edge solution, pushing the boundaries of chromatographic capabilities in environmental and analytical sciences.

b) Gel permeation chromatography

Gel Permeation Chromatography (GPC) has emerged as a valuable sample cleanup technique in the context of dioxin analysis, offering an effective means of separating complex mixtures and removing interfering matrix components. GPC is particularly well-suited for samples with a high degree of complexity, such as those found in environmental matrices. In the cleanup process, the sample is fractionated based on molecular size as it passes through a column packed with porous beads. Larger molecules, including unwanted matrix constituents, are excluded and elute earlier, while smaller target analytes, such as dioxins, pass through the column more slowly. This differential elution allows for the isolation and concentration of dioxins, simplifying the subsequent analytical steps. GPC-based cleanup provides a selective and reproducible approach, enhancing the precision of dioxin analysis by minimizing interference from co-extracted compounds. As environmental regulations continue to demand stringent monitoring of dioxins, GPC-based sample cleanup stands as a robust technique contributing to the reliability and accuracy of analytical results in environmental and biological samples (Tuinstra et al., 1994).

c) Evolution of automated sample preparation systems

Automated dioxin sample preparation systems represent a transformative leap in analytical workflows, streamlining the complex and meticulous process of sample preparation for dioxin analysis. These sophisticated systems leverage advanced robotics, precise liquid handling, and cutting-edge technologies to enhance efficiency, reproducibility, and overall laboratory productivity. By automating tedious sample preparation steps such as extraction, cleanup, and derivatization, these systems significantly reduce the potential for human error and ensure consistency in results. The integration of automated dioxin sample preparation systems not only accelerates the analytical process but also minimizes the risk of contamination, thereby improving the accuracy of dioxin measurements. This technological innovation not only saves time and resources but also empowers laboratories to handle higher sample throughput, making it

an invaluable tool for environmental monitoring, food safety assessments, and various research endeavors focused on dioxin analysis (Abad et al., 2000)

1.7.3. Quantification

a) Screening methods

The utilization of CALUX (Chemical-Activated LUciferase gene eXpression) assays for bioanalytical screening of dioxins has become a valuable approach in evaluating dioxin contamination across diverse environmental and biological samples. These assays rely on genetically modified cell lines containing a luciferase reporter gene controlled by dioxin-responsive elements (DREs). Upon exposure to dioxins or dioxin-like compounds, these cells produce luciferase enzyme, whose luminescent output can be measured. This bioluminescent signal correlates directly with the concentration of dioxins in the sample. CALUX assays offer several advantages over traditional analytical methods, including swift results, heightened sensitivity, and specificity for dioxin-like compounds. They also serve as a cost-effective screening tool for large-scale monitoring initiatives, facilitating the evaluation of dioxin contamination in various environmental matrices, food items, and biological specimens. Furthermore, CALUX assays complement conventional chemical analysis techniques by providing insights into the biological activity of dioxins, enhancing our understanding beyond mere chemical quantification. In summary, CALUX-based bio analytical screening plays a pivotal role in dioxin risk assessment and management, contributing significantly to safeguarding human health and environmental integrity against the detrimental effects of dioxin exposure (Hoogenboom et al., 2006).

b) Confirmatory methods

Gas chromatography coupled with high-resolution mass spectrometry (GC-HRMS) is widely regarded as the gold standard for dioxin analysis due to its exceptional mass precision, capable of measuring up to four decimal points, and its ability to conduct unbiased quantitation at ultra-trace levels. However, despite its accuracy, the technique's high capital and operational costs, as well as handling difficulties, have hindered its widespread acceptance. In 2014, the European Union recognized GC-MS/MS as a confirmatory method for dioxin analysis in food and feed matrices. GC-MS/MS follows a similar procedure to GC-HRMS, with the sample undergoing

gas chromatographic separation followed by ionization and fragmentation in the mass spectrometer. Unlike GC-HRMS, GC-MS/MS employs two mass analyzers arranged in series. The first mass analyzer selects precursor ions based on their mass-to-charge ratio (m/z) and fragments them into smaller product ions. These product ions are then analyzed in the second mass analyzer to generate a tandem mass spectrum. By selecting specific precursor and product ions, GC-MS/MS achieves exceptional sensitivity and selectivity, enabling the detection and quantification of dioxins at trace levels in complex samples. This method is particularly beneficial for samples with high matrix interference, as the tandem mass spectrometer can selectively measure the target analytes while minimizing interference from background compounds. However, it's crucial to note that sample cleanup is essential for unbiased quantitation, as this step removes co-extracts and concentrates the analytes of interest. Studies have shown that GC-MS/MS-based methodologies can perform comparably to GC-HRMS when coupled with a rigorous sample cleanup protocol (Reiner., 2010). Despite its cost-effectiveness and ease of operation, achieving matrix-specific validation in accordance with stringent EU regulations remains a challenge for GC-MS/MS methodology. **Fig 1.7** shows schematic representation of GC-MS/MS working while **Fig 1.8** compares various techniques according to sensitivity, selectivity and speed.

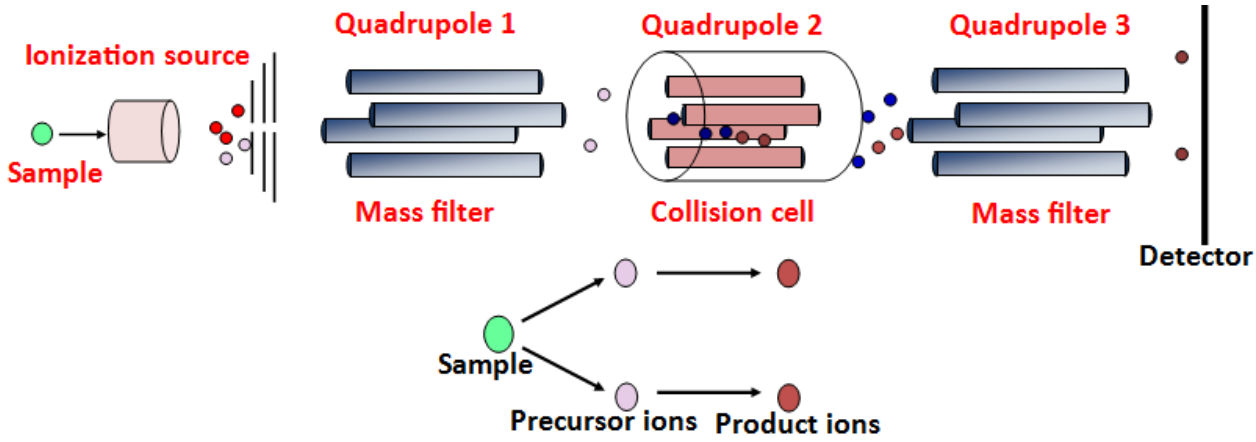


Fig.1.7 Schematic representation of GC MS-MS based quantification of analytes

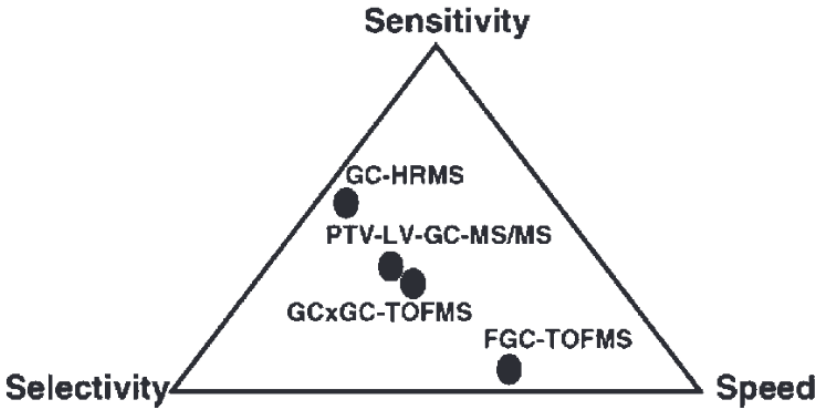


Fig.1.8 Schematic representation comparison of different quantification instruments in dioxin analysis in terms of selectivity, sensitivity and speed (Reiner, 2010)

1.8. Global challenges in wide spreading surveillance

a) Analytical challenges

The global surveillance of persistent organic pollutants (POPs) faces intricate analytical challenges, particularly in the context of monitoring dioxins and PCBs, which are present in food and feed matrices at remarkably low concentrations, often in the picogram range. Analyzing these ultra-trace levels demands the utilization of highly specialized and unbiased analytical methodologies. Cutting-edge instruments such as Gas Chromatography-High Resolution Mass Spectrometry (GC-HR MS) or Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS) are essential for achieving the required sensitivity and specificity in detection. Adding to the complexity is the diversity in the composition of matrices across different types of samples. The unique characteristics of each matrix necessitate the development of analytical methodologies that are tailored to their specific attributes. Recognizing the potential for biased quantification results due to the intricacies of matrix composition, regulatory bodies are actively involved in establishing stringent method validation criteria. The goal is to ensure not only the accuracy and reliability of analytical outcomes but also the comparability of results across diverse matrices.

This meticulous scrutiny and emphasis on validation contribute to the credibility of global surveillance efforts, assuring stakeholders that the reported data accurately reflects the true extent of dioxin and PCB contamination. In essence, the successful global surveillance of these persistent pollutants hinges on the development and adherence to advanced and matrix-specific analytical protocols.

b) Lack of global strategies

The absence of globally coordinated strategies for the surveillance of dioxins and PCBs poses significant challenges to understanding and mitigating the impact of these persistent organic pollutants on a global scale. Dioxins and PCBs, being highly mobile and persistent in the environment, can easily cross national borders through air and water currents. The lack of a standardized and harmonized approach to monitoring allows for inconsistencies in surveillance methodologies and reporting standards among different countries. This fragmentation in surveillance efforts makes it difficult to obtain a comprehensive and accurate picture of the prevalence and distribution of dioxins and PCBs globally. Additionally, varying regulatory frameworks and enforcement mechanisms can lead to disparities in the management and control of these contaminants.

The interconnectedness of global trade and industrial processes further emphasizes the need for collaborative strategies. A shared, standardized approach would facilitate the exchange of information, data, and best practices among nations. This, in turn, would enable a more effective response to emerging contamination threats, ensuring the protection of ecosystems, wildlife, and human health. To address this gap, there is a pressing need for increased international cooperation and the development of unified frameworks for the surveillance of dioxins and PCBs. This could involve the establishment of common monitoring protocols, data-sharing mechanisms, and joint research initiatives. Such global collaboration is crucial to creating a cohesive and robust response to the challenges posed by these persistent pollutants, transcending geographical boundaries for the collective benefit of the environment and public health.

b) Elevated analysis cost

The global surveillance of dioxins and PCBs faces a considerable obstacle due to the elevated costs associated with their analysis. One of the primary contributors to this financial challenge is

the necessity for advanced confirmatory analyte quantification instruments such as Gas Chromatography-High Resolution Mass Spectrometry (GC HR MS). The high initial investment and ongoing maintenance costs for these sophisticated instruments make them financially burdensome for many countries, particularly those with limited resources. Moreover, the expense extends to the sample preparation stage, where the use of commercially available ready-to-use sample clean-up cartridges incurs additional costs. Furthermore, the need for highly skilled personnel to operate and maintain these instruments adds to the overall cost of analysis. Training professionals in the operation and interpretation of results from these specialized instruments requires further investments in education and skill development.

Addressing the challenge of elevated analysis costs is crucial for promoting more widespread and equitable global surveillance efforts. Initiatives focused on technological innovation, cost reduction strategies, and collaborative agreements for resource-sharing can contribute to making these critical analytical tools more accessible to a broader range of countries. By mitigating the financial barriers associated with dioxin and PCB analysis, the international community can foster a more inclusive and comprehensive approach to monitoring these persistent organic pollutants, ensuring the protection of global environmental and public health.

1.9. Scope and objectives of the thesis

The scope of this thesis represents a multifaceted approach to addressing the challenges associated with monitoring Persistent Organic Pollutants (POPs), particularly focusing on Dioxins and Poly Chlorinated Biphenyls (PCBs). A significant aspect involves the development and optimization of a novel analytical method utilizing Gas Chromatography coupled with Tandem Mass Spectrometry (GC-MS/MS) for the precise quantification of dioxins and PCBs in fish and fish oil matrices. Through meticulous validation procedures conforming to stringent regulatory standards, the objective is to establish a robust and reliable protocol capable of providing accurate confirmatory analysis at reduced costs compared to traditional methods. Furthermore, the research extends to field studies aimed at assessing the environmental impact of POPs in a historical hotspot region. By analyzing fish and sediment samples collected from this area, the study aims to elucidate contamination levels and potential sources of precursor species, thereby contributing valuable insights into environmental pollution pathways and associated risks to human health. Additionally, the investigation explores the utilization of activated carbon

derived from renewable sources, such as coconut shells, as a versatile sorbent material for sample cleanup and decontamination. By synthesizing cleanup materials and fabricating user-friendly sample cartridges, the research endeavors to streamline sample preparation processes and enhance efficiency in POPs surveillance. Overall, this thesis aims to advance analytical methodologies, foster sustainable practices, and provide practical solutions for effective monitoring and management of POPs contamination in environmental and food matrices. In essence the broad objectives of the thesis are as follows.

- Develop and validate a cost-effective GC-MS/MS protocol for quantifying dioxins and PCBs in fish & fish oil matrices.
- Assessment of POPs hotspot with the developed methodology
- Integrate low-cost dioxin and PCBs analysis through the synthesis and fabrication of indigenous clean-up columns.
- Establish a sustainable decontamination strategy in animal feed matrices

Chapter

2

Development and critical validation of GC-MS/MS based analytical protocol for confirmatory quantification of dioxins and PCBs in fish/ fish oil

2.1. Abstract

Developments in the field of gas chromatography-tandem mass spectrometry (GC-MS/MS) led to its acceptance as a confirmatory tool for the quantification of dioxins and PCBs in food and feed matrices. Compared to the gold standard magnetic sector high-resolution mass spectrometer, this technique is inexpensive, easy to use and requires less maintenance. Regulatory agencies set stringent validation criteria for GC-MS/MS based methods to achieve superior efficiency through unbiased results. The present study focused on the development of a GC-MS/MS based complete analytical workflow and its in-depth validation in fish and fish oil matrices according to, European Union regulation (EU) 644/2017. Sample preparation steps such as extraction and clan-up have been systematically optimized to ensure minimal analyte loss and efficient removal of co-extracts. A calibration curve based method incorporating sample size and final constitutional volume was employed to determine the limit of quantification of individual congeners. The obtained $\sum TEQ_{WHO\text{-}2005}$ at LOQ level in the case of dioxins is 0.363 pg TEQ/g wet weight (fish matrix), 0.727 pg TEQ/g (fish oil matrix) and the obtained value is less than $1/5^{th}$ of maximum level (ML) established for both fish and fish oil matrices. The performance of the method was evaluated in terms of precision and accuracy by conducting spike recovery experiments. The measurement uncertainty, which is decisive in the compliance assessment, was evaluated in accordance with the EU regulation for individual congeners in both matrices. In short, the present study could set a benchmark that establishes a cost effective validated analytical workflow for confirmatory analysis of dioxins and PCBs in two critical food/feed matrices of interest.

2.2. Introduction

Dioxins and polychlorinated biphenyls (PCBs) contamination incidents, such as the Guar gum contamination originated from India and the Belgium crisis, have underscored their profound impact on human health and economic stability (Wahl et al., 1998). These incidents highlight the urgent need for global surveillance of these contaminants due to their adverse effects, even at ultra-trace levels. However, the widespread monitoring of dioxins and PCBs faces challenges, particularly due to the high analysis costs associated with confirmatory analysis using sophisticated instruments like gas chromatography coupled with high-resolution magnetic sector mass spectrometry (GC- sector HRMS). In response to these challenges, recent advancements in

mass spectrometry have positioned gas chromatography-tandem mass spectrometry (GC-MS/MS) as a cost-effective and reliable alternative for targeted analysis of dioxins and PCBs. Studies have demonstrated convincingly that GC-MS/MS yields comparable results to GC-sector HRMS, offering attractive features such as lower installation costs and reduced operational and maintenance requirements. Recognizing these advantages, international organizations like the European Union (EU) and the United States Environmental Protection Agency (USEPA) have endorsed GC-MS/MS, establishing various criteria for its validation in the confirmatory analysis of dioxins and PCBs (Franchina et al., 2019). Despite this recognition, there is still a noticeable lack of studies in this field, particularly concerning complete matrix-specific method validation.

This chapter strategically emphasizes the significance of monitoring two representative vital food and feed matrices viz. fish and fish oil. Fish, due to its continual interaction with sediment the ultimate repository of contaminants, and its widespread consumption as a dietary staple, underscores the importance of surveillance of these contaminants within this matrix. Similarly, fish oil, being a fat-rich medium extensively used in feed formulations, presents an additional concern due to its propensity to accumulate lipophilic contaminants. Regulatory bodies have meticulously identified specific food and feed materials, establishing analytical protocols and maximum permissible levels to safeguard the food supply chain. Delving into regulatory frameworks, EU regulation (EC) No 589/2014 marks a crucial milestone as the first regulation to acknowledge GC-MS/MS-based methods as a confirmatory analytical protocol for dioxins and PCBs (European Union, 2017). Subsequently, the European Union recommended the Performance-Based Measurement System (PBMS) approach, empowering laboratories to develop their own validated analytical methodologies.

This research critically assesses each criterion outlined in the repealed version of (EC) No 589/2014, now (EU) 2017/644, examining criteria applicable to general analytical methodologies, GC-based methods, and GC-MS/MS methods (European Union, 2017). The chapter scrutinizes the efficacy of the developed methodologies, encompassing not only GC-MS/MS-based quantification but also crucial sample preparation steps. Integral to the confirmatory analytical methodology is the isotope dilution mass spectrometry (IDMS) methodology, which utilizes ^{13}C labeled internal standards to estimate analytes loses in the

sample preparation phase of the analysis. The present work systematically optimizes sample extraction and clean-up steps, achieving optimum internal standard recovery. Additionally, the measurement uncertainty of the developed method, a decisive factor in compliance assessment, is thoroughly estimated, considering various uncertainty contributions in both fish and fish oil matrices.

In essence, this research critically validates the developed GC-MS/MS-based analytical methodology in two widely consumed food and feed matrices: fish and fish oil. The cost-effective methodology and validation plan established in this study have broad applicability, providing a foundation for extrapolation into different matrices of interest.

2.3. Materials and methods

2.3.1. Chemicals and consumables

GC headspace grade hexane, toluene and dichloromethane solvents used in the sample extraction and subsequent clean–up steps were obtained from Spectrochem, India. Solvent standard based GC-MS/MS calibration is achieved using standard combination of analytes obtained from Cambridge Isotopic Laboratories (CIL), Massachusetts, USA. Labeled internal and recovery standards used in sample fortification and instrument calibration were obtained from CIL and Wellington Labs. Final reconstitution of analyte fractions were carried out using n-Nonane as keeper solvent. Sodium sulphate and HYDROMATRIX^{TM} used in the sample extraction step were obtained from Sigma Aldrich and Agilent Technologies respectively. The consumables for the sample cleanup step such as multilayer silica; alumina and carbon columns were obtained from FMS Inc, USA as ready to use cartridges. In the GC-MS/MS system, ultra-high purity helium and nitrogen gases (99.999%, Bhoruka, India) were used as carrier gas and collision gas, respectively.

2.3.2. Analyzed sample details

The method validation experimental trials were performed with fish and feed grade fish oil samples. Comparatively dioxin/PCBs free samples wherein the congener concentrations were either undetected or well below 1/5th of maximum level were used to assess the accuracy of the method through spike recovery experiments. To assess the performance of the method, background and cross contamination possibilities a representative matrix, i.e., pork fat is used as

the quality control material, taking into account its long term stability. Furthermore the efficacy of the developed method was demonstrated by participating in international proficiency test in fish matrix.

2.3.3. Optimization of sample preparation

Fish samples were lyophilized prior to the extraction step to remove moisture content. For a typical analysis, sample uptake was 10 g wet weight of fish tissue. The freeze-dried fish samples were extracted using both classical Soxhlet extraction and pressurized liquid extraction techniques and compared efficiency of each extraction technique in terms of internal standard recovery. Fish oil samples (2.5 g per experiment) were taken directly for the cleanup step as it is 100% fat matrix. In a typical extraction procedure, the lyophilized fish tissue mixed with HYDROMARIX^{TM} was weighed and spiked with ^{13}C- labeled congeners of PCDD/Fs and dl-ndl PCBs. For Soxhlet based extraction the ISTD spiked sample was carefully transferred into a cellulose thimble and the loaded thimble placed in the Soxhlet flask. The Soxhlet extraction conditions such as cycles/hr., extraction time, extraction solvent were maintained according to the EPA-8290 standard. Accelerated solvent extraction cell was set up by introducing neutral silica layer below the sample layer to enable in line clean-up. The extraction parameters of ASE such as pressure, oven temperature, static time, no of static cycles etc have been optimized. The extracts obtained from both extraction techniques were concentrated using a rotary evaporator. The concentrated extract was completely transferred to the sample holding tube of cleanup system. The column chromatographic technique based on multi-layer silica, activated carbon and alumina was used for effective sample clean-up. Sample clean-up was performed in a semi-automated system (EZprep123^{TM}, FMS, USA) capable of vacuum assisted solvent elution. The series-connected sorbent cartridges loaded with concentrated extract after conditioning step. The entire assembly was eluted with an optimized volume of n-hexane. The trapped PCDD/Fs and coplanar PCBs in the carbon column were reverse eluted as fraction 1, and MO-ndl PCBs were reverse eluted as fraction 2 from the alumina column.

2.3.4. GC-MS/MS analytical method (Instrument parameters, instrument calibration)

The quantification of dioxins and PCBs were conducted using Dioxin analyzer consists of 7890 B gas chromatograph equipped with 7690 A automatic liquid sampler and 7000C triple quadruple mass spectrometer (Agilent Technologies, USA). **Table 2.1** represents the GC and

MS/MS parameters corresponding to fraction 1 (Dioxins & NO PCBs) & and fraction 2 (MO & NDL-PCBs). Effective separation of congeners was achieved using classic DB-5 MS 60 m × 250 µm x 0.25 µm (Agilent) capillary column. Solvent standard based calibration was carried out, which consist of 9 calibration points (1: 2 serial dilutions). In each calibration point the concentration of internal standard and recovery standard were kept constant.

Table 2.1 Optimized GC-MS/MS analytical method

Quantification of analytes	
Instrument: Gas chromatograph coupled with tandem in space triple quadruple mass spectrometer Gas chromatograph System: 7890 GC & 7690 A Automatic Liquid Sampler, Agilent Technologies Triple quadruple mass spectrometer: 7000C, Agilent Technologies	
Optimized analytical method- Gas chromatograph	
Capillary column specification	Agilent DB-5 MS, 60 m × 0.25 mm, 0.1 µm
Capillary column pneumatics	Constant flow (0.98 mL/min, He carrier gas)
Liner	5190-2293 Ultra-Inert, single taper
Injection mode	Solvent vent mode
Injection volume	4 µL
Inlet Temperature program	120^0C for 0.08 min, 100^0C/min to 160^0C, hold 0.15 min, 700^0C/min to 330^0C, hold 8 min, 10^0C/min to 250^0C
Oven temperature program	60^0C for 1 min, 30^0C/min to 270^0C, hold 1 min, 2^0C/min to 310^0C, 10^0C/min to 325^0C, hold 5 min
Optimized analytical method- MS-MS	
Ionization mode	EI 70 eV
Acquisition mode	MRM (Two precursors are two specific product ions)
Interface temperature	300^0C
Ion source temperature	330^0C
Solvent delay	11 min
Quadruple temperature	Q1 (150^0C) Q2 (150^0C)

2.3.5. Method validation

According to EU Regulation 644/2017, the developed GC-MS/MS methodology must meet criteria across three distinct categories: basic requirements, specific requirements for GC-MS-based methods, and specific criteria for GC-MS/MS-based confirmatory methods. **Table 2.2**

outlines the various criteria specified in the regulation alongside the corresponding criteria employed for method validation. The estimation of measurement uncertainty plays a crucial role in compliance assessment, with EU-established methodologies mandated for trace level analysis of dioxins and PCBs. This study critically evaluates various potential sources of uncertainty, incorporating them into the calculation of the total uncertainty budget. **Table 2.3** presents the various factors considered and the overall methodology adopted to calculate the measurement uncertainty.

Table 2.2
Method validation overview

Validation criteria as per EU regulation 644/2017	Methodology adopted
Basic requirements to be met by analytical methodology for dioxins and PCBs	
1. Establishment of Limit of Quantification • For dioxins the detectable quantities should be upper femtogram (10^{-15} g) , low picograms (10^{-12} g) for dl-PCBs and for ndl-PCBs in the nanogram range (10^{-9} g) • The calculated TEQ at LOQ level should be 1/5 th of the maximum level	A calibration curve based methodology was adopted to estimate the LOQ considering both sample size and reconstitution volume. **Equation 1** was utilized for LOQ calculation, where LACP represents the Lowest Acceptable Calibration Point and SD denotes the Standard Deviation. The LOQ values calculated individually for each congener were utilized to estimate the TEQ value at the LOQ level and ensure compliance with regulatory criteria. $\boldsymbol{Limit\ of\ quantification\ (LOQ)}\left(\frac{pg}{g}\right) = (\boldsymbol{LACP} + 3 * \boldsymbol{SD})\left(\frac{pg}{\mu l}\right) \times \frac{\boldsymbol{Sample\ reconstitution\ volume}\ (\mu l)}{\boldsymbol{Sample\ Size}\ (g)}$ .. **Eq 2. 1**
2. High selectivity • Methodology should be capable to remove the co extracts for efficient quantification of analytes • Differentiation of analytes into different fractions to achieve un biased quantitation	The optimized cleanup methodology, utilizing multilayer silica, alumina, and activated carbon-based column chromatography, achieves effective removal of co-extracts and fractionates analytes into two distinct fractions based on the planarity of the congeners. Fraction 1: planar PCDD/Fs and NO-PCBs Fraction 2: non-planar MO-PCBs and NDL-PCBs Confirmation of co-extract removal was verified through spike recovery experiments and attainment of noise-free chromatograms.

3. High accuracy (Trueness and Precision)

Accuracy of the developed method should demonstrate through spike recovery experiments carried out at maximum level, ML, ML/2 and 2 ML.

- Trueness: The variance between the measured mean value of an analyte in a spiked sample and its theoretical value, presented as a percentage of the theoretical concentration. The threshold trueness limit is ± 20%.

- Precision: Expressed in terms of the relative standard deviation derived from spike recovery experiments at ML results obtained under reproducibility conditions. The threshold trueness limit is < 15 %.

Conducted spike recovery experiments at ML, ML/2, and 2 ML levels using the optimized analytical methodology, and assessed trueness and precision using Equations 2 and 3. Evaluated compliance with the threshold values specified in the regulation.

$$Trueness(\%) = \frac{Mean\ value\ measured - Certified\ value\ of\ CRM}{Certified\ value\ of\ CRM} X100$$

--- Eq 2.2

Precision as $RSD_R = \frac{SD_{replicate\ analysis}}{|Mean\ value|} \times 100$--------**Eq 2.3**

4. Quality control (QC –chart)

Recording and checking of control sample analyses shall ensure that the analytical performance aligns with the specified requirements**.**

Regular quality control experiments were conducted using a pork fat matrix, and the resulting data was utilized to establish quality control curves for different congener groups. This curve serves the purpose of monitoring background contamination and assessing the performance of the developed methodology over time.

Specific criteria that must be fulfilled GC-MS-based methods	
5. Acceptable difference between upper bound and lower bound values The acceptable difference between upper bound and lower bound levels should be less than 20 % for confirmation of exceedance of maximum or action levels.	De facto, this criterion in the regulation will be satisfied once the calculated TEQ level at the LOQ is less than 1/5th of the maximum level (criterion 1).
6. Internal standard recovery In the confirmatory analytical methodology, the recovery of ^{13}C-labeled internal standards for all individual congeners spiked before the extraction/cleanup of the sample must be monitored. - The recoveries of internal standards should fall within the range of 60 to 120%. However, lower or higher recoveries for individual congeners are acceptable provided their contribution to the TEQ value does not exceed 10% of the total TEQ value (based on the sum of PCDD/F and dioxin-like PCBs). - The method should utilize recovery standards or surrogate standards before GC-MS analysis to calculate the recovery rates of internal standards.	The current methodology employs an isotope dilution mass spectrometric approach, utilizing 13C-labeled internal standards of all native analytes, to monitor analyte recovery. The following equations were utilized to calculate various parameters and assessed compliance with regulatory limits. *Native relative response factor,* $RRF_{native} = \frac{A_{native} \times Q_{ISTD}}{Q_{native} \times A_{ISTD}}$ **.....Eq 2.4** *ISTD relative response factor,* $RRF_{ISTD} = \frac{A_{ISTD} \times Q_{RSTD}}{Q_{ISTD} \times A_{RSTD}}$ **.....Eq 2.5** $Average\ relative\ response\ factor, \overline{RRF} = \frac{1}{m}\sum_{i=1}^{m} RRF_i$ **.....Eq 2.6** *Internal standard recovery* $\%\ ISTD_{REC} = \frac{A_{RSTD} \times Q_{rs} \times 100}{A_{RSTD} \times Q_{ISTD} \times \overline{RRF}}$ **-----Eq 2.7** Where, A= Instrument response Q=Concentration ISTD = Internal Standard RSTD=Recovery Standard TEF =Toxicity Equivalent Factor

7. Removal of interfering substances - Gas chromatographic separation of isomers (difficult to achieve separation in capillary gas chromatographic column) must be adequate (with less than 25% peak-to-peak between 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF. - For NDL-PCBs relative retention time in relation to internal standards, threshold limit is ± 0,25 %).	The developed methodology uses an efficient 60 m capillary column coated with (5%-phenyl)-methylpolysiloxane is used to achieve analyte separation in the gas chromatograph. Following equations were used to calculate various parameters and assessed compliance with regulatory limits $Peak\ resolution\ \% = (1 - \frac{2v}{h_1+h_2}) \times 100$-----Eq 2.8 $Relative\ Retention\ Time\ = \frac{Target\ compound\ \ retention\ time}{Related\ aaISTDs\ reltention\ time}$----**Eq 2.9** $RRT\ Deviation = \frac{RRT_{Calibration\ point/sample} - \overline{RRT}_{Calibration}}{\overline{RRT}_{Calibration}}$……..**Eq 2.10** Where h1, h2 adjacent peak heights and v is the valley height.
8. Calibration with standard curve The range of calibration should cover maximum/ action levels	A ten point calibration curves were constructed for both fraction 1 and 2, covering action/ maximum level. The efficacy of the calibration was evaluated by monitoring the coefficient of determination R^2 value.
Specific criteria that must be fulfilled GC-MS/MS based methods	
9. MRM transitions monitoring Method should monitor two specific precursor ions and each with one specific product ion	The developed methodology monitors two precursor ions and two product ions for each analyte, considering the loss of COCl (dioxins) and Cl_2 (PCBs) during fragmentation.
10. Ion ratio monitoring The permitted tolerance of relative ion intensity is ± 15%	Calculated ion ratio using following equations for individual calibration points and real sample analysis and assessed compliance with regulatory limits

	$Ion\ ratio = \frac{Quantifier\ response}{Qualifier\ response}$…..**Eq 2.11** $\text{I}on\ ratio\ bias = \frac{Ion\ ratio_{calib\ point/sample} - Ion\ ratio_{mean\ calibration}}{Ion\ ratio_{mean\ calibration}} \times 100$..**Eq 2.12**
11. Quadruple mass resolution Unit or better mass resolution	Each quadruple is maintained in unit mass resolution, to distinguish masses with 1 Da offset.

Table 2.3

Measurement uncertainty estimation- Overview

Uncertainty Contribution	Methodology/ Equations used to evaluate
	Major contributors
1. Intermediate precision	Used to address the possible random error. The associated uncertainty u_{rw} is calculated from the data of repeated spike recovery experiments (at ML level). $Relative\ intermediate\ precision\ standard\ uncertainty, u_{Rw,rel} = \frac{S_{Rw}}{\bar{x}}$---**Eq 2.13** Where S_{Rw} is the standard deviation and $\bar{x}$ is the average result obtained.
2. Method bias (trueness)	Method bias uncertainty is calculated from the above mentioned precision experiments, considering uncertainty associated with the fortified concentration The following equations are used to estimate the uncertainty contribution.

	$RMS_{bias,fort} = \sqrt{\frac{\sum(bias_{fort,rel,i})^2}{n}}$---**Eq 2.14** $u_{fort} = \sqrt{u_{conc}^2 + u_{vol}^2}$....*Eq 2.15* $u_{bias,fort,rel} = \sqrt{RMS_{bias,fort}^2 + u_{fort}^2}$....**Eq 2.16**
	Minor contributors
3. Calibration curve uncertainty	Covers the uncertainty related to instrument calibration (GC-MS/MS). It is calculated by considering standard deviation associated with relative response factor of calibration points (section 6, Method validation overview) $u_{cal,rel} = \frac{s_{RRF,rel}}{\sqrt{n}}$.....**Eq 2.17**
4. Volume uncertainty	The methodology encompasses uncertainty contributions from glassware, micro-pipettes, measuring cylinders, etc., utilized for dispensing specific volumes. Certified uncertainty values (±a) are employed for each case, assuming a rectangular distribution to calculate the uncertainty contribution. The relative uncertainty ($u_{v,rel}$) is determined by dividing the uncertainty value (u_v) by the volume amount. Subsequently, the uncertainty contributions associated with volume are aggregated to yield the standard uncertainty of volume. $u_v = \frac{a}{\sqrt{3}}$.....**Eq 2.18**
5. Standard solution concentration uncertainty	Uncertainty related to the concentration of native and internal standard ($u_{st,\ rel}$) used to calibrate instrument and sample fortification. The corresponding

	uncertainty contributions can obtain from "COA(Certificate of Analysis) of CRMs.
6. Uncertainty related to weighing	Related to uncertainty contribution of weighing instrument. It can be obtained from routine calibration certificate of balance obtained through regular external calibration procedure. Represented as $u_{w,rel}$
Combining major and minor contributions to calculate combined expanded measurement uncertainty	
Combined uncertainty, $u_{c,rel}$	The above mentioned uncertainty contributions were combined to obtain the combined uncertainty. $u_{c,rel} = \sqrt{u_{Rw,rel}^2 + u_{bias,rel}^2 + u_{cal,rel}^2 + u_{v,rel}^2 + u_{st,rel}^2 + u_{w,rel}^2}$ ….**Eq 2.19**
Combined uncertainty in terms of TEQ	$u_{TEQ} = \sqrt{\sum_{i=1}^{29}(TEF_i x\, u_{c,rel})^2}$…..**Eq 2.20**
Expanded Measurement Uncertainty	Calculated expanded measurement uncertainty by multiplying combined uncertainty with the coverage factor, k $U = k \times u_{TEQ}$……**Eq 2.21** Developed methodology used a coverage factor of 2 corresponding to level of confidence of 95%.

2.4. Results and discussion

2.4.1. Optimized sample extraction

Fig.2.1 depicts the classic Soxhlet extraction and Pressurized Liquid Extraction (PLE) methods. In this study, these two extraction techniques were fine-tuned specifically for extracting the fish matrix. Given that the fish oil matrix consists entirely of fat in liquid phase, fish oil samples were taken directly to the sample cleanup step, bypassing the conventional extraction procedure. In a standard Soxhlet extraction procedure, the lyophilized fish sample, spiked with ^{13}C labeled internal standards of all analytes, was loaded into a cellulose thimble and positioned within the extraction flask. The optimized methodology employs a hexane: Dichloro Methane (DCM) mixture in a 1:1 ratio as the extraction solvent. Following this, the extraction flask, equipped with a reflux condenser, and a round bottom flask containing the extraction solvent were assembled and placed onto a heating mantle. The optimized conditions involved maintaining a rate of four to five siphons per hour and conducting a continuous sixteen-hour extraction process. Utilizing the PLE system can significantly reduce extraction time due to elevated temperatures that greatly enhance extraction efficiency. Applying pressure with a nitrogen cylinder aids in maintaining the extraction solvent in its liquid phase even at high temperatures within the PLE cell oven. In a typical PLE experiment, fish samples spiked with internal standard (ISTD) were loaded into a stainless steel extraction cell. Layers of sodium sulfate and silica were introduced to remove moisture content and facilitate the reduction of sample cleanup load, respectively (**Fig. 2.2**). The extraction cell was filled to 90-95% capacity using inert diatomaceous earth as a filler material. Optimized PLE parameters included a cut-off pressure of 1700 psi, an oven temperature of 130°C, a static time of 5 minutes, three static cycles, 80% rinse volume, and a 5-minute nitrogen purge time. Both extraction techniques demonstrate high efficiency in extracting analytes, yielding optimal internal standard recovery rates (section 2.4.4). The PLE system, capable of reducing extraction time and cleanup load through inline cleanup, proves to be the more efficient extraction methodology. Although subsequent studies predominantly utilize PLE-based extraction procedures, optimizing the Soxhlet extraction method is found to be highly beneficial for seamlessly conducting extraction procedures during downtime of the PLE instrument in the laboratory.

2.4.2. Optimized sample clean up

Fig2.3. illustrates the column assembly and the corresponding solvent elution workflow for the cleanup of dioxins/PCBs samples. The setup involves a series of cartridges including multi-layer silica, activated carbon, and alumina, which facilitate the removal of co-extracts and fractionation of congeners into two fractions based on their planarity. Sequential reactions occur in the multi-layer silica column, with the analytes subsequently eluted into the activated carbon. The activated carbon selectively traps planar analytes (PCDD/Fs and non-ortho PCBs), while the alumina column traps mono-ortho and non-dioxin-like PCBs respectively. A critical challenge in this process is optimizing the solvent volumes (hexane and toluene) during different elution steps. This optimization aims to achieve optimal recovery of internal standards, removal of interfering substances, and proper fractionation of analytes. One challenge specific to non-ortho PCBs (NO-PCBs) is that increasing the forward elution volume (hexane) can cause NO-PCBs to elute onto the alumina column, while reducing the forward elution volume may result in NO-PCBs not eluting from the multi-layer silica column. (MO and NDL PCBs). Reverse elution of the activated carbon and alumina cartridges with toluene yields fraction 1 (PCDD/Fs & non-ortho PCBs) and fraction 2 (MO & NDL PCBs) For instance, **Fig. 2.4 (a)** indicates that a 220 ml hexane elution resulted in low recovery of NO-PCBs, but optimizing the forward elution volume to 180 ml yielded optimum recovery of NO-PCBs. Through a series of trial and error experiments, different elution volumes were optimized for both fish and fish oil matrices. The optimized elution volumes for these matrices are provided in **Fig. 2.4(b)**.

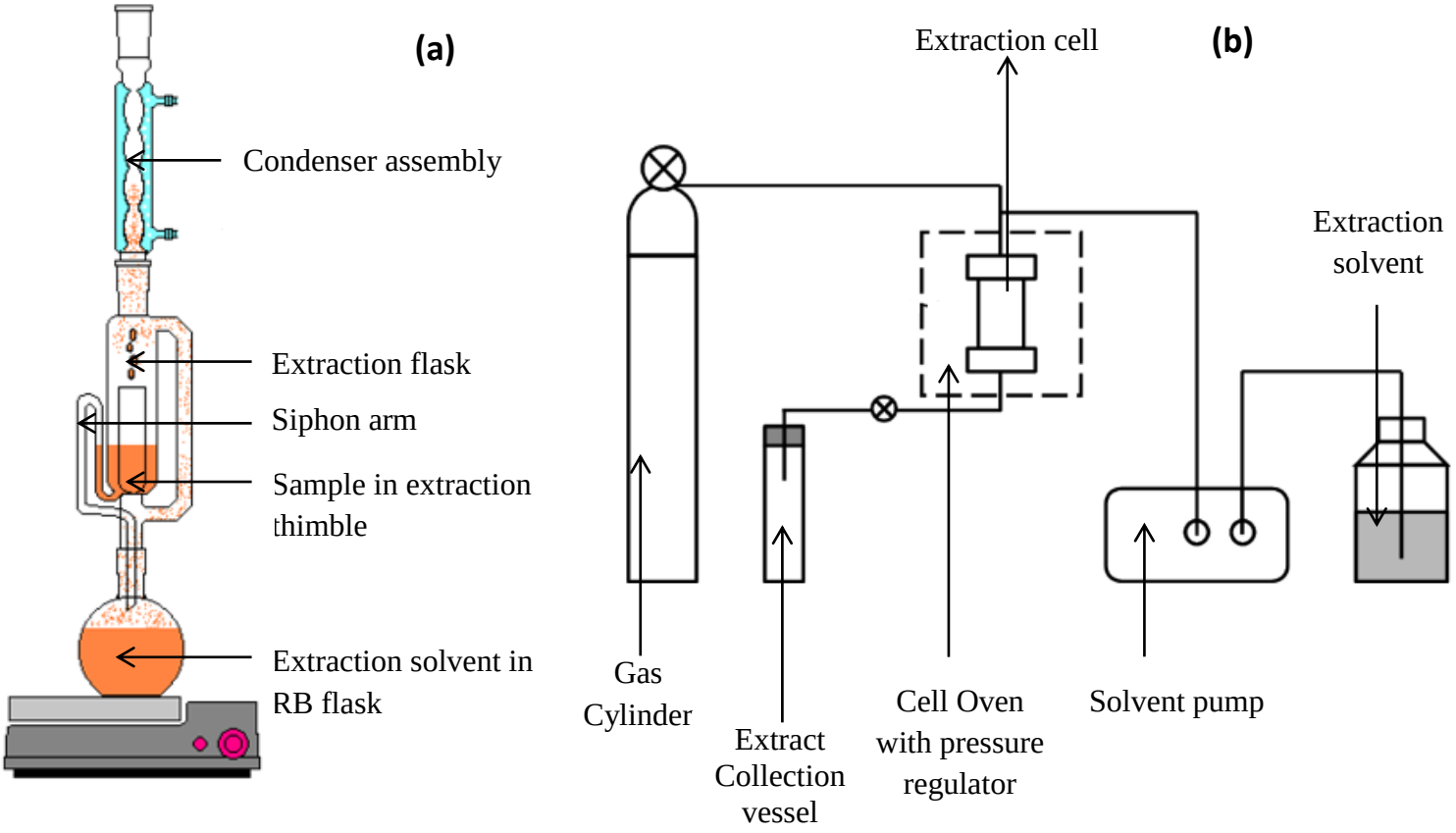


Fig. 2.1 Schematic representation of sample extraction procedure (a) Classic Soxhlet extraction (b) Pressurized Liquid Extraction (PLE)

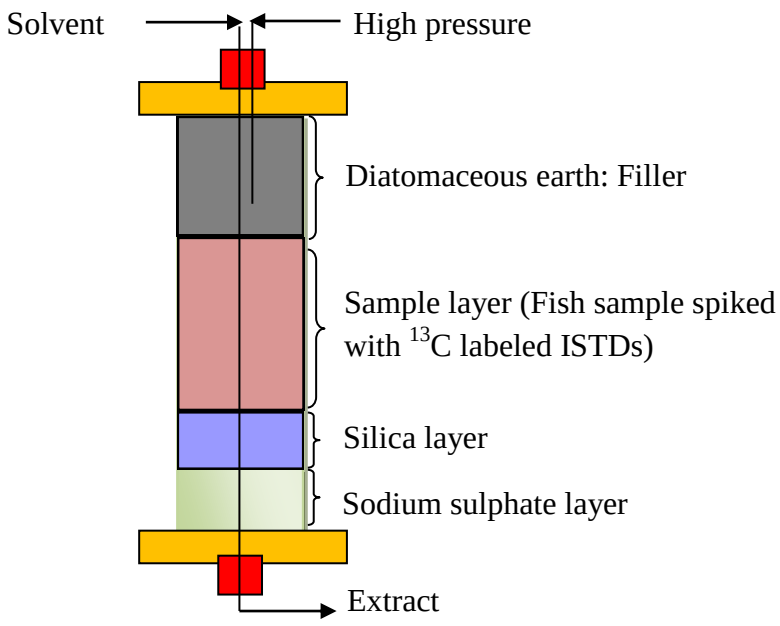


Fig. 2.2 Schematic representation of the composition of the PLE extraction cell

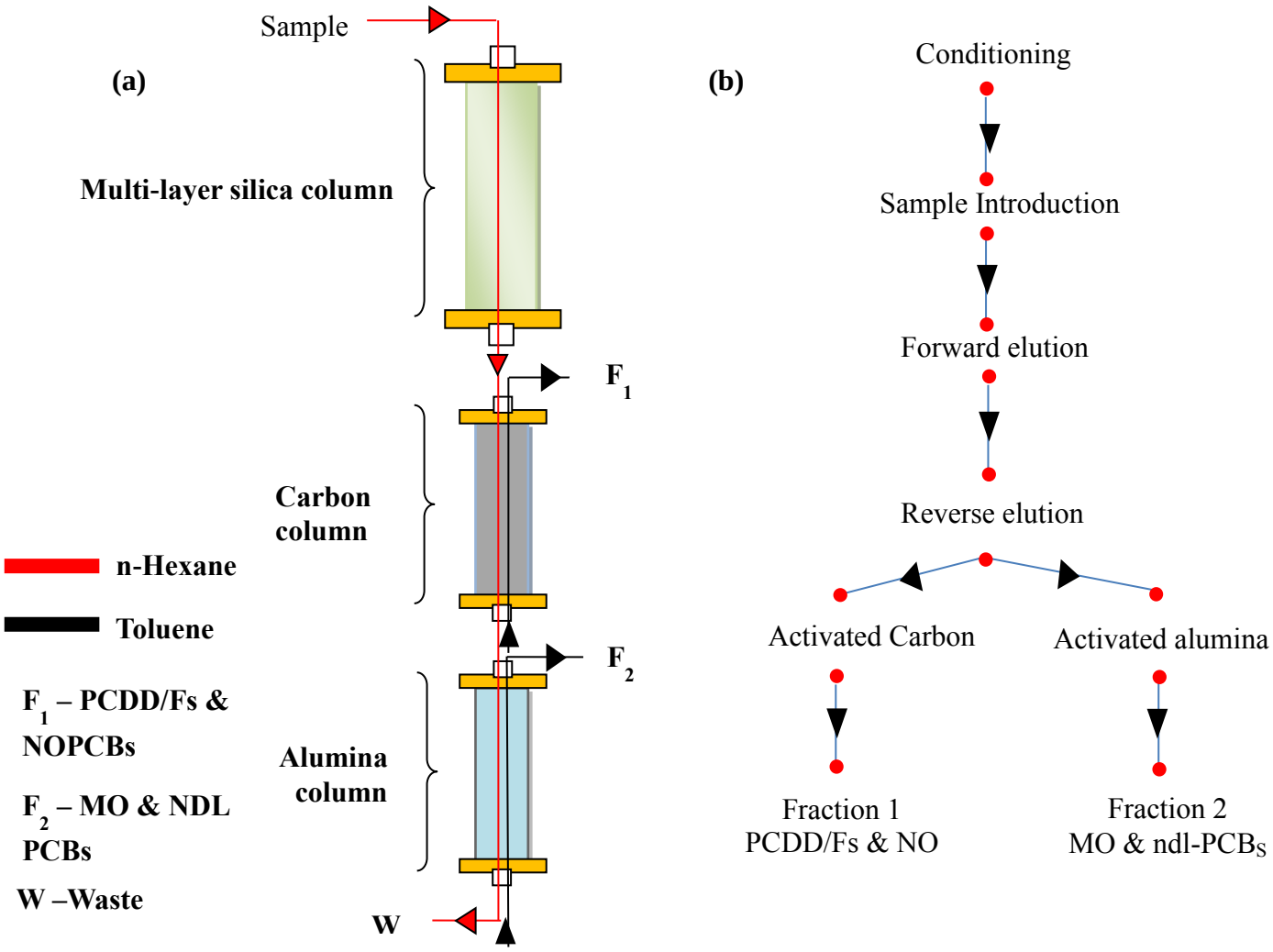


Fig. 2.3 Dioxin/PCBs sample cleanup **(a)** Chromatographic column assembly and**(b)** elution workflow

2.4.3. GC-MS/MS instrument calibration and limit of quantification

Calibration curves for two fractions, fraction 1 (PCDD/Fs & NO PCBs) and fraction 2 (MO & ndl-PCBs), were established using a solvent standard-based method. Ten calibration levels were created through a 1:2 serial dilution process. Throughout all calibration levels, concentrations of internal standards and recovery standards remained constant. The concentrations of congeners corresponding to each level were given in **Table 2.4**, the range of calibration is selected in such a way that to cover maximum and action level stipulated for fish and fish oil matrix by EU regulation. The effectiveness of instrument calibration was evaluated using the determination

coefficient value (R^2) for each congener. **Table 2.5** presents the R^2 values for individual congeners, all of which exceeded 0.999, indicating high calibration efficiency.

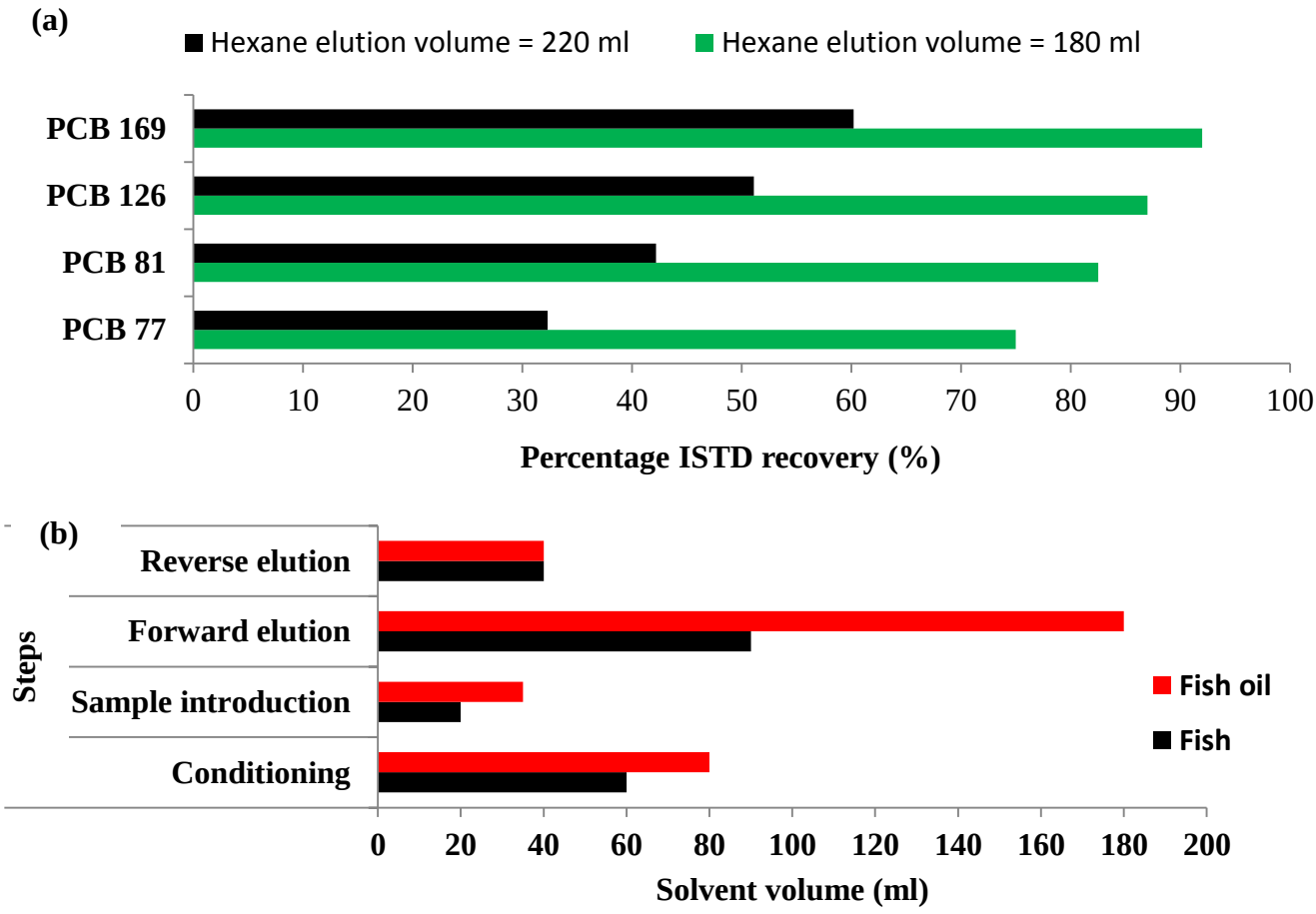


Fig.2.4 (a) NO-PCBs recoveries with different elution volumes of n-hexane **(b)** Optimized elution volumes in fish oil and fish matrices

Earlier GC-sector HRMS based method considered signal/ noise (S/N) ratio to estimate the limit of quantification (LOQ) values of dioxins/PCBs congeners. But GC-MS/MS works in selective ion monitoring, S/N ratio based LOQ estimation will lead to unrealistic values, which cannot be achieve in the real matrices. Focant *et al* proposed procedural blank based LOQ estimation, which consider blank levels of congeners and standard deviation blank levels repeated six times. This approach fails in estimating LOQ of congeners when its procedural blank level is zero. Since dioxin analysis utilizes thorough sample clean-up, present study proposes a calibration

curve based estimation of the LOQ. The Eq (2.22) is used to estimate the LOQ values considering both sample size and the reconstitution volume. The Lowest Acceptable Calibration Point (LACP) is selected in such a way that the lowest calibration level of the congener which satisfies four critical criteria namely deviation to average RRF (<30%), Relative Standard Deviation (RSD) of response factors ± 15 %, Relative ion intensities ≤ 15%.and the correct Retention time window. After selecting the LACP, eight replicate injection of this level was carried out and three times calculated standard deviation is added to LACP. Finally this value is multiplied and divided with sample reconstitution volume and sample size in order to get LOQ values in the pg/g unit. The obtained LOQ values were given in the **table 2.5.**

$$Limit\ of\ quantification\ (LOQ)\ \left(\frac{pg}{g}\right) = (LACP + 3 * SD)\left(\frac{pg}{\mu l}\right) \times \frac{Sample\ reconstitution\ volume\ \ (\mu l)}{Sample\ Size\ (g)} \ldots (\mathbf{Eq\ 2.22})$$

The regulatory criteria related to LOQ necessitates that the TEQ calculated at LOQ level should be less than 1/5th of the maximum permissible level assigned for that matrix. Present study calculated the TEQ at LOQ levels and the obtained results were less than 1/5th of the maximum level in both fish and feed grade fish oil matrices.

Table 2.4
Calibration overview

Category	Concentration in pg/ml									
	CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	CAL 7	CAL 8	CAL 9	CAL 10
TCDD/Fs	5	10	20	40	80	160	320	640	1280	2560
Pe-HpCDD/Fs	12.5	25	50	100	200	400	800	1600	3200	6400
OCDD/Fs	25	50	100	200	400	800	1600	3200	6400	12800
13C-T-HpCDD/Fs	500	500	500	500	500	500	500	500	500	500
dl-PCBs	39.1	78.1	156.2	312.5	625	1250	2500	5000	10000	20000
NDL-PCBs	39.1	78.1	156.2	312.5	625	1250	2500	5000	10000	40000
13C-PCBs	500	500	500	500	500	500	500	500	500	500
13C-OCDD/Fs	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Recovery standards	250	250	250	250	250	250	250	250	250	250

Table 2.5
Calibration efficiency and limit of quantification

SI NO	Compounds	TEF	R^2 values	LACP (pg/ml)	Calculated LOQ	
					Fish matrix	Fish oil matrix
1	2378-TCDD	1	0.9996	31.0	0.078	0.155
2	2378-TCDF	0.1	0.9992	25.0	0.063	0.125
3	12378-PeCDD	1	0.9995	52.9	0.132	0.265
4	12378-PeCDF	0.03	0.9998	40.0	0.100	0.200
5	23478-PeCDF	0.3	0.9995	39.8	0.100	0.199
6	123478-HxCDD	0.1	0.9993	85.9	0.215	0.430
7	123789-HxCDD	0.1	0.9996	69.6	0.174	0.348
8	123678-HxCDD	0.1	0.9991	75.1	0.188	0.376
9	123678-HxCDF	0.1	0.9994	64.5	0.161	0.322
10	123789-HxCDD	0.1	0.9993	67.1	0.168	0.336
11	123789-HxCDF	0.1	0.9993	35.1	0.088	0.175
12	234678-HxCDF	0.1	0.9992	46.8	0.117	0.234
13	1234678-HpCDD	0.01	0.9993	42.7	0.107	0.213
14	1234678-HpCDF	0.01	0.9991	53.3	0.133	0.267
15	1234789-HpCDF	0.01	0.9947	27.7	0.069	0.138
16	OCDF	0.0003	0.9994	141.3	0.353	0.706
17	OCDD	0.0003	0.9998	153.5	0.384	0.768
	∑PCDD/Fs TEQ at LOQ level				**0.363**	**0.727**
18	PCB 77	0.0001	0.9994	87.6	0.219	0.438
19	PCB 81	0.0003	0.9995	88.3	0.221	0.441
20	PCB 126	0.1	0.9996	129.3	0.323	0.646
21	PCB 169	0.03	0.9996	108.5	0.271	0.542
22	PCB-123(MO)	0.00003	0.9997	121.4	0.304	0.607
23	PCB-118(MO)	0.00003	0.9999	101.5	0.254	0.508
24	PCB-114(MO)	0.00003	0.9996	95.6	0.239	0.478
25	PCB-105(MO)	0.00003	0.9996	105.0	0.263	0.525
26	PCB-167(MO)	0.00003	0.9998	74.8	0.187	0.374
27	PCB-156(MO)	0.00003	0.9999	95.0	0.237	0.475
28	PCB-157(MO)	0.00003	0.9998	68.9	0.172	0.344
29	PCB-189(MO)	0.00003	0.9999	86.1	0.215	0.431
	∑dl-PCBs TEQ at LOQ level				**0.041**	**0.081**
	∑PCDD/Fs + dl-PCBs TEQ at LOQ level				**0.404**	**0.808**
30	PCB-28(ind)	NA		87.3	4.363	
31	PCB-52(ind)			81.5	4.077	
32	PCB-101(ind)			83.0	4.152	
33	PCB-153(ind)			104.3	5.214	

34	PCB-138(ind)	61.3	3.067
35	PCB-180(ind)	84.2	4.209
	∑NDL-PCBs at LOQ level		**25.1**

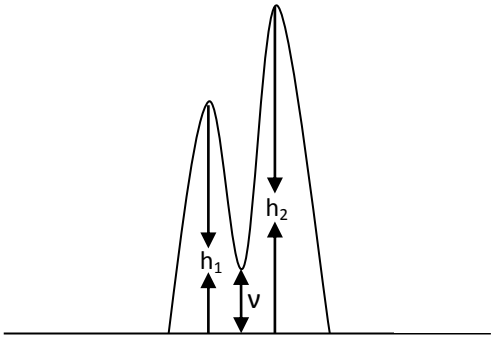
Concentration unit: pg/g wet weight (fish matrix) and pg/g relative to a feed with 12 % moisture content

Maximum levels fish matrix	Maximum levels fish oil matrix
PCDD/Fs: 3.5 pg TEQ/g wet weight	PCDD/Fs: 5 pg TEQ/g wet weight
PCDD/Fs+dl PCBs: 6.5 pg TEQ/g wet weight	PCDD/Fs+dl PCBs: 20 pg TEQ/g wet weight
NDL PCBs: 75 ng/g weight	NDL PCBs: 175 ng/g weight

2.4.4. Removal of interfering substances and control of recoveries

The efficacy of the quantification method for PCDD/Fs and PCBs depends on its ability to accurately differentiate ultra-trace level analytes from co-extracts, which are present in much higher concentrations within a particular matrix. Regulatory standards necessitate the implementation of appropriate cleanup procedures to eliminate co-extracts and to segregate analytes into distinct groups for easier quantification. The European Union (EU) imposes additional criteria for the quantification method of PCDD/Fs–DL PCBs and NDL PCBs, focusing on the identification and confirmation of analytes. For PCDD/Fs and DL-PCBs, gas chromatographic separation of isomers is required to have less than 25% peak-to-peak difference between specific isomer pairs (1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF). As for NDL PCBs, relative retention time (RRT) in relation to internal or reference standards should deviate no more than ± 0.25%. In our developed method, gas chromatographic separation of analytes within each fraction was conducted using a DB-5 MS 60 m capillary column. The performance of this column was validated by assessing the separation of closely eluting isomers, such as 2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF. Two parameters, % valley and peak resolution, were calculated for these congeners across ten calibration points. % Valley measures the height of the valley relative to the average height of the two peaks (**Fig. 2.5**), with values consistently below 10%, meeting regulatory criteria. Peak resolution indicates the degree of separation between peaks, with values consistently above 90%, indicating effective chromatographic separation (**Table 2.6**). For NDL PCBs, identification and confirmation were achieved by calculating the mean RT from calibration data for all congeners and assessing the deviation from this mean during both calibration and routine sample analysis. The results complied with the permissible deviation criterion of ± 0.25% as mandated by regulation (**Table 2.7**). The effectiveness of the extraction and clean-up procedures can be evaluated by examining the recoveries of internal standards. **Fig.**

2.7 illustrates the recovery rates of internal standards obtained from spike recovery tests (ML experiment) conducted on fish and fish oil matrices using the developed methodology. The recovery rates fell within the range of regulatory limit of 60-120%. Overall, the developed method satisfies regulatory requirements for the quantification of PCDD/Fs and PCBs, demonstrating effective separation, control of analyte recovery and accurate identification and confirmation of analytes.



$$Valley\ \% = \frac{2v}{h_1 + h_2} \times 100$$

Fig 2.5 Valley % calculation

Table 2.6
Peak resolution and % valley

Calibration level	**1,2,3,4,7,8-HxCDF (h_1)**	**1,2,3,6,7,8-HxCDF (h_2)**	**Valley (v)**	**Peak resolution (%)**	**% valley**
Cal 1	257	971	0	100.0	0.00
Cal 2	524	551	5	99.1	0.93
Cal 3	961	1068	27	97.3	2.61
Cal 4	1422	1337	31	97.8	2.25
Cal 5	3689	3821	371	90.1	9.88
Cal 6	5982	6085	521	91.4	8.63
Cal 7	16185	14750	959	94.0	6.00
Cal 8	29737	27056	1896	93.3	6.67
Cal 9	60176	55099	3622	93.7	6.28
Cal 10	123456	104567	4125	96.4	3.62
ML experiment	4956	3956	321	92.8	7.20

Table 2.7

RRT deviation of NDL PCBs in calibration and ML experiment

Compounds	RRT Deviation (%)										
	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6	Cal7	Cal8	Cal9	Cal10	ML
PCB-28	-0.02	0.01	-0.02	0.01	0.01	0.01	-0.02	0.01	0.01	-0.02	0.01
PCB-52	0.01	-0.02	-0.02	0.01	-0.02	0.01	0.01	0.01	0.01	0.01	-0.02
PCB-101	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.04	0.00
PCB-153	0.01	-0.02	-0.01	-0.01	0.01	0.01	-0.01	0.01	0.01	-0.01	-0.01
PCB-138	0.00	0.02	0.00	-0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PCB-180	-0.02	0.00	0.00	0.00	0.00	0.00	-0.02	0.00	0.00	0.00	0.00

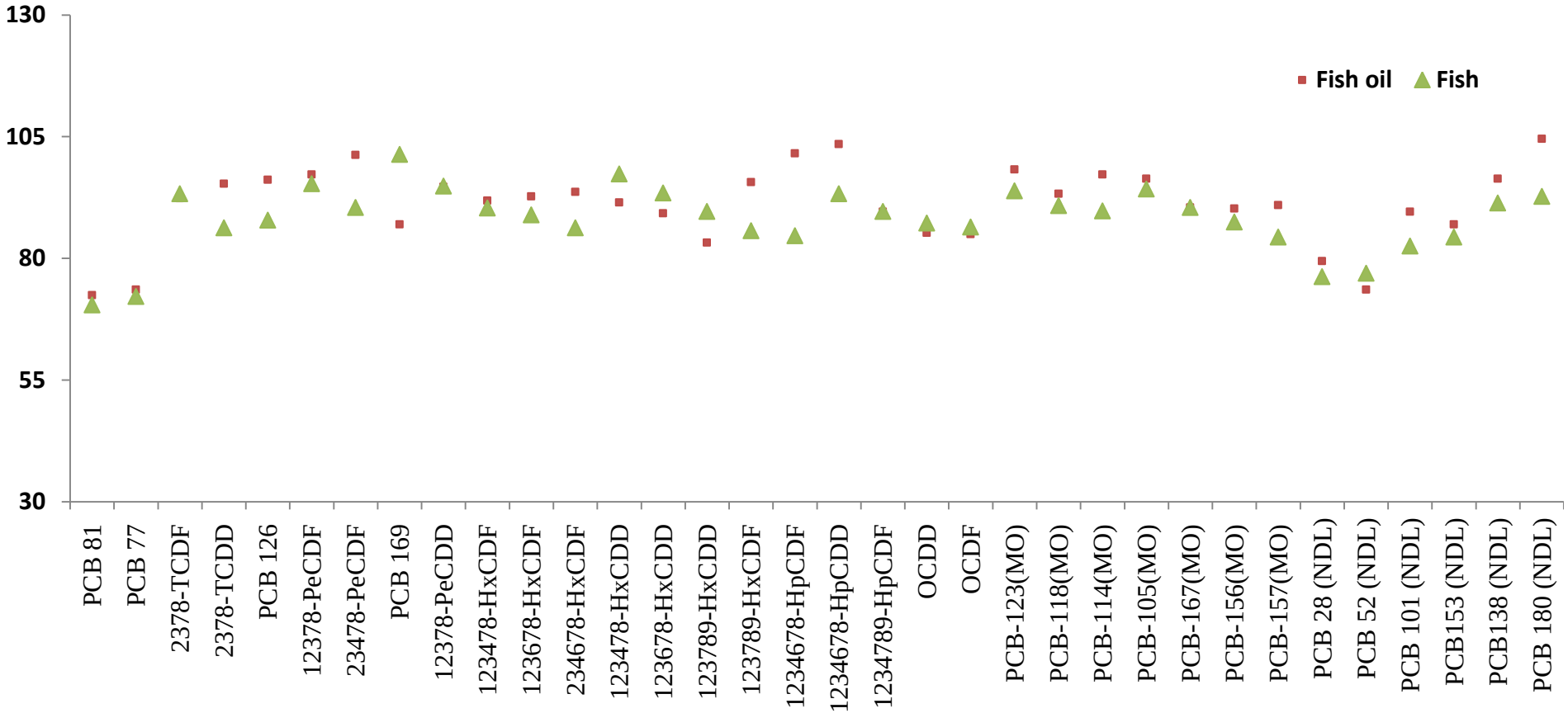


Fig 2.7 Obtained recovery rates of ^{13}C- ISTDs in ML experiment of fish and fish oil matrices

2.4.5. Method validation at maximum level and general quality control measures

According to EU regulations, a screening/confirmatory method for detecting dioxins/PCBs in a specific matrix needs to demonstrate by conducting spike recovery experiments at regulated maximum levels (ML/2, ML, 2ML). These experiments evaluate the method's performance in terms of both precision and accuracy. Fish and fish oil matrix with analytes levels < LOQ, confirmed through prior experiments were used to conduct the spike recovery experiments to demonstrate method precision and accuracy. In the case fish matrix the maximum levels established are PCDD/Fs: 3.5 pg TEQ/g wet weight; PCDD/Fs+dl PCBs: 6.5 pg TEQ/g wet weight NDL PCBs: 75 ng/g weight, while in the case of feed grade fish oil are PCDDFs: 5 pg TEQ/g; PCDD/Fs+dl PCBs: 20 pg TEQ/g; NDL PCBs: 175 ng/g (relative to a feed with 12% moisture content). Both fish and fish oil matrices fortified with native analytes at ML/2, ML, 2ML levels were analyzed using optimized sample extraction and clean-up methodology as per sections 2.4.1 and 2.4.2 respectively. In each levels repeated experiments n=8 times. The obtained data from these experiments were evaluated to determine both method precision and accuracy. According to EU regulation accuracy of the method is calculated as bias % ie, difference between the mean value measured for an analyte in a matrix spiked at ML/2, ML, 2ML levels and the theoretical (fortified value) expressed as percentage. The calculated bias % (method accuracy) should be in the bracket of – 20 % to + 20 %. **Fig 2.8** represents the calculated value of method accuracy (% bias) in both fish and fish oil matrices, and the obtained values were in accordance with the regulatory criteria. The replicate analysis results were used to evaluate the precision of the developed method, according to the regulation the method precision should evaluate in terms of the relative standard deviation (RSD_R) calculated from replicate analysis experiment data obtained under reproducible conditions and the threshold limit is < 15%. The evaluated precision values as RSD_R were < 15 % (**Fig 2.8**) for both fish and fish oil matrices. The obtained data demonstrates the efficacy of the developed analytical methodology both in terms of precision and accuracy.

The efficacy of the optimized method was further demonstrated by constructing QC chart for all analyte groups. Laboratory uses pork fat matrix in order to carry out regular QC experiments considering long term stability of it. 2.5g of pork fat mixed with diatomaceous earth was extracted and analyzed after entire analytical procedure. Experiments were repeated in regular intervals to generate QC curve. Quality control chart was constructed in such way that, lines

corresponding to mean value and mean value ± 2 SD (considering 95 % confidence) were drawn and plotted results obtained from individual quality control experiment. Obtained results in the case of each analyte groups were varied in between mean ± 2 SD. No experiment results were exceeded this limit which shown high efficacy of the developed method. The obtained QC charts for each group of analytes were given in **Fig 2.9**

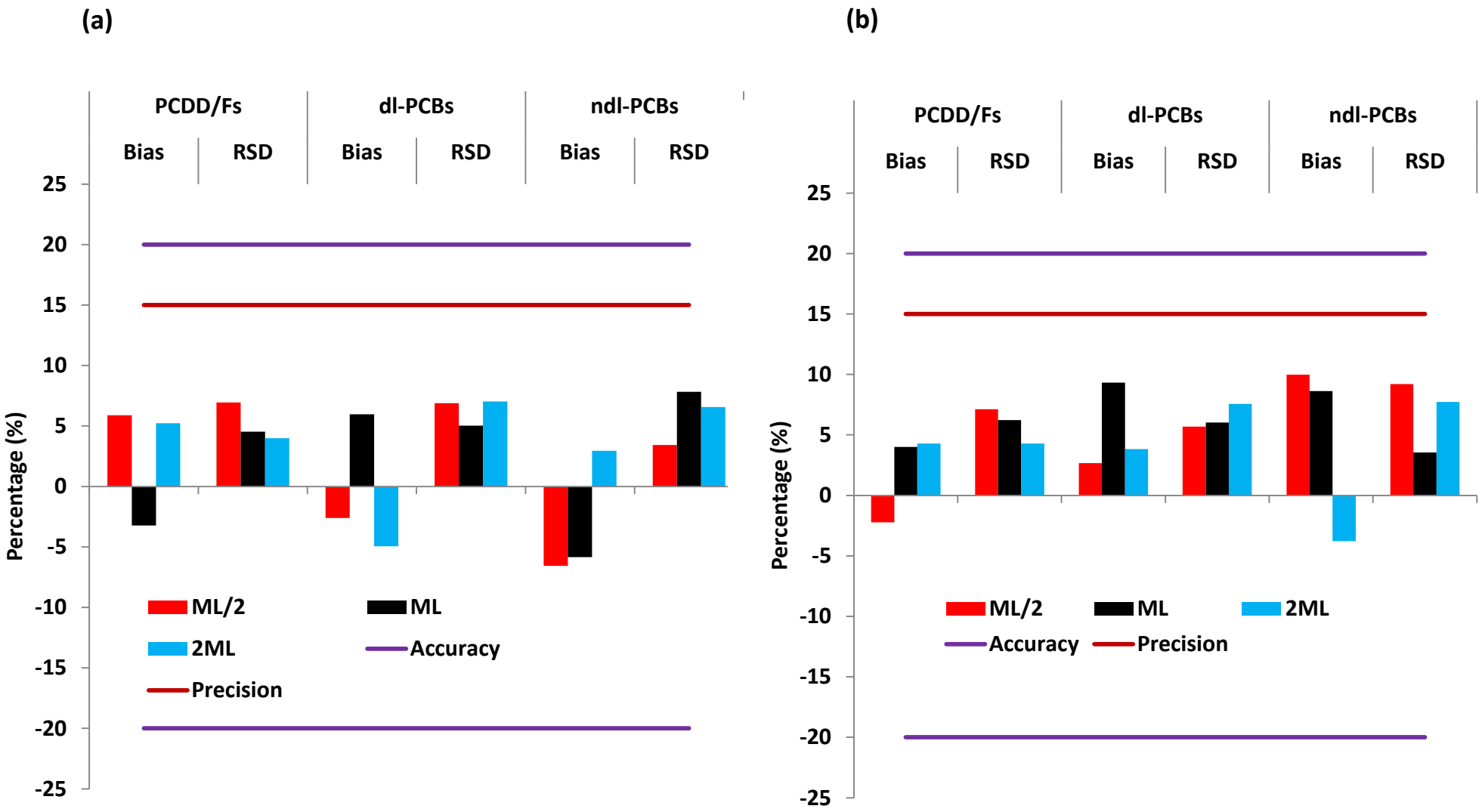


Fig 2.8. Spike recovery experiment results (a) fish matrix (b) fish oil matrix

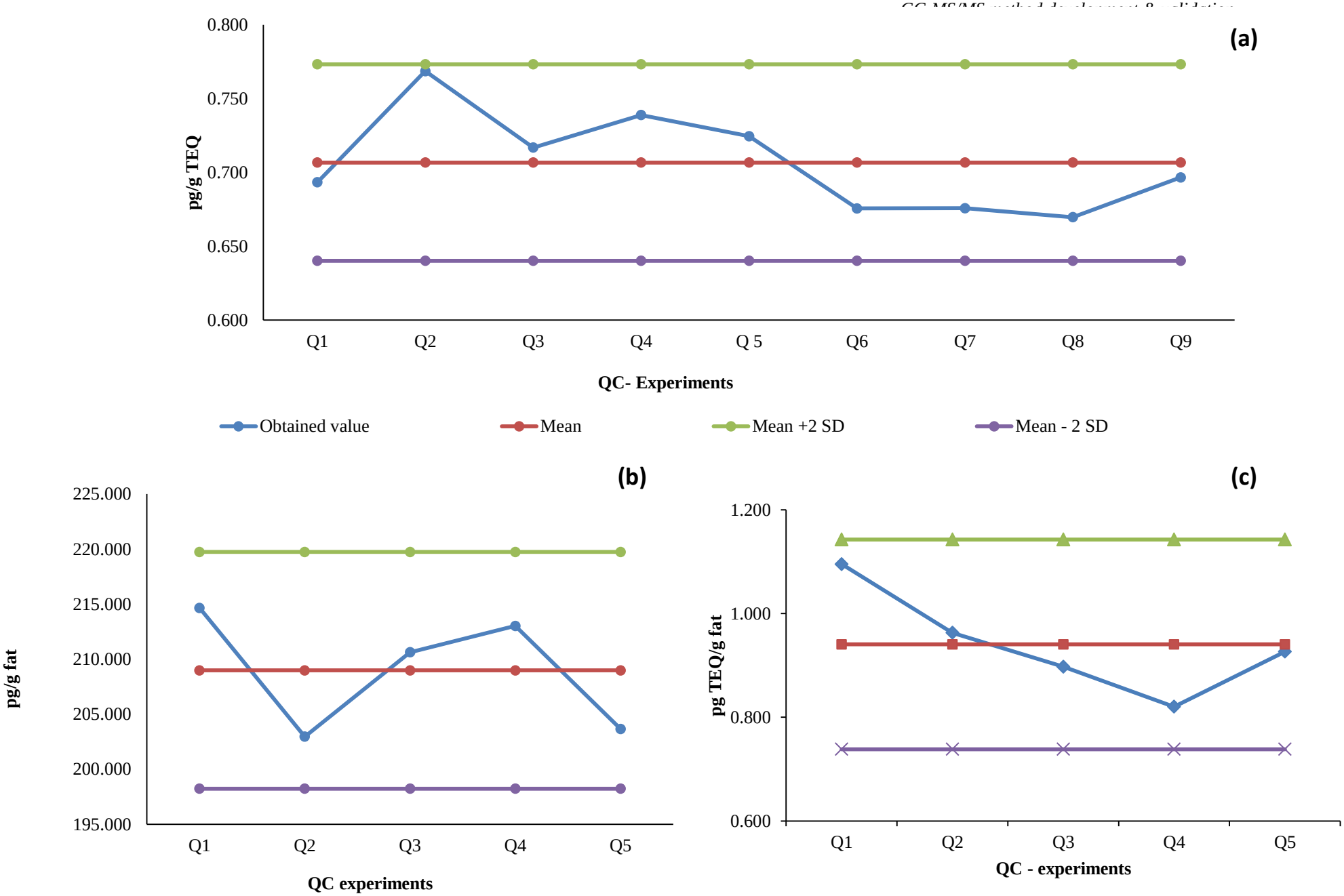


Fig 2.9. Constructed QC charts (a) PCDD/Fs (b) NDL-PCBs (C) dl-PCBs

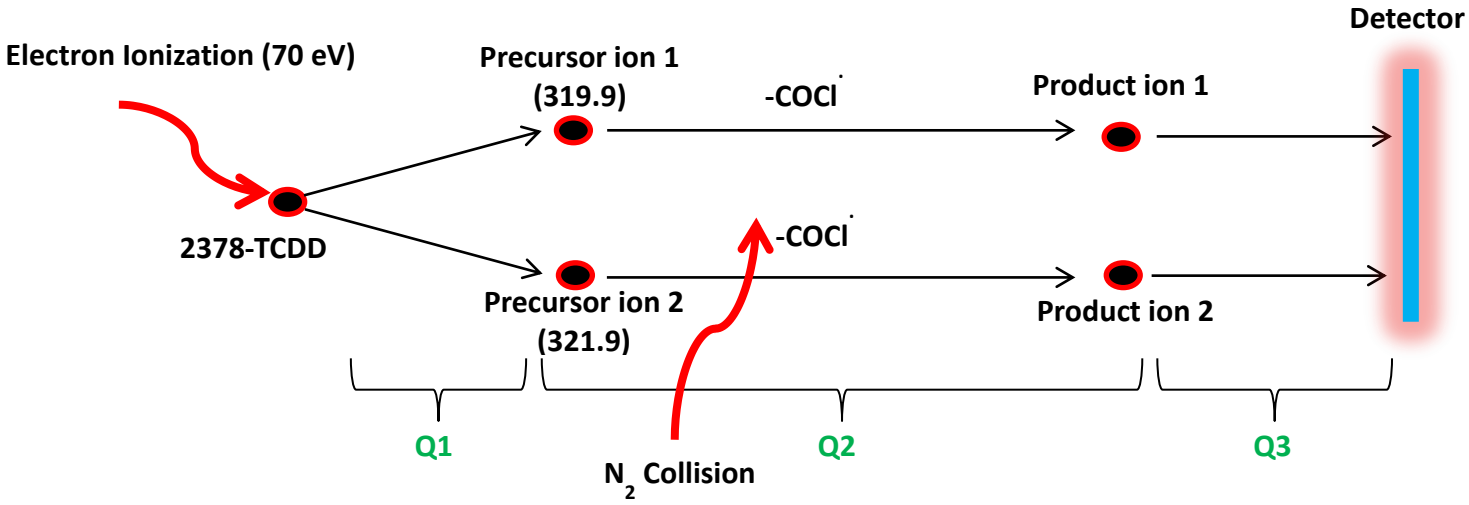
2.4.6. MRM monitoring, quadruple resolution and relative ion intensity tolerance

In compliance with EU Regulation 644/2017, a confirmatory methodology employing GC-MS/MS is required to monitor two distinct precursor ions, each with specific transition product ions for both dioxins and PCBs. The methodology devised strictly adheres to this mandate by meticulously monitoring these ions (2 precursor and 2 product ions per analyte) using multiple reaction monitoring (MRM) mode in the mass spectrometer. Selection of precursor ions and their corresponding product ions (Quant and Qual transitions) was guided by the analyte's molecular weight and unique fragmentation pattern. Dioxins exhibit a characteristic fragmentation pattern marked by the loss of a COCl moiety, either through sequential loss of Cl and CO or concentrated loss of COCl, whereas PCBs demonstrate specific loss of chlorine moiety. A schematic depiction in **Fig. 2.10** illustrates the ionization process of 2378-TCDD at each stage during the MRM mode mass experiment. Furthermore, **Table 2.8** presents the monitored masses of precursor and product ions for both native and labeled analytes. To ensure unbiased quantification, the methodology maintains each quadruple resolution to unit, enabling the spectrometer to distinguish between ions with a one Dalton offset, as mandated by EU Regulation 644/2017. Ion ratio serves as a crucial parameter in GC-MS/MS analysis, playing a pivotal role in verifying analyte identity and assessing analysis reliability. This ratio is computed by considering instrument response corresponding to both quantifier and qualifier. As per regulatory stipulations, the acceptable tolerance in relative ion intensity should be <15%. The bias in ion ratio/relative ion intensity tolerance is determined. **Fig. 2.11** presents the bias values obtained for spike recovery experiments at maximum level for both fish and fish oil matrices. Results demonstrating <15% tolerance underscore the efficacy of the developed methodology in achieving unbiased quantitation of analytes.

Table 2.8.
Monitored mass transitions

Category	Compounds	Native Compounds				^{13}C - Internal Standards			
		Quant transition		Qual transition		Quant transition		Qual transition	
		Precursor 1	Product 1	Precursor2	Product 2	Precursor 1	Product 1	Precursor2	Product 2
PCDD/Fs	**2378-TCDD**	319.9	256.9	321.9	258.9	331.9	268.9	333.9	270.9
	2378-TCDF	303.9	240.9	305.9	242.9	315.9	252.9	317.9	254.9
	12378-PeCDD	355.9	292.9	357.9	294.9	367.9	304.9	369.9	306.9
	PeCDFs (2)	339.9	276.9	341.9	278.9	351.9	288.9	353.9	290.9
	HxCDDs (3)	389.8	326.9	391.8	328.9	401.8	338.9	403.8	340.9
	HxCDFs (4)	373.8	310.9	375.8	312.9	385.8	322.9	387.8	324.9
	1234678-HpCDD	423.8	360.8	425.8	362.8	435.8	372.8	437.8	374.8
	HpCDFs (2)	407.8	344.8	409.8	346.8	419.8	356.8	421.8	358.8
	OCDD	457.7	394.8	459.7	396.8	469.7	406.8	471.7	408.8
	OCDF	441.7	378.8	443.7	380.8	453.7	390.8	455.7	392.8
NO-PCBs	**PCB 77, 81**	289.9	219.9	291.9	221.9	301.9	231.9	303.9	233.9
	PCB 126	323.9	253.9	325.9	255.9	335.9	265.9	337.9	267.9
	PCB 169	359.9	289.9	361.9	291.9	371.9	301.9	373.9	303.9
MO PCBs	**PCB 123, 118, 114,105**	325.9	255.9	327.9	257.9	337.9	267.9	339.9	269.9
	PCB 189	393.8	323.8	395.8	325.8	405.8	335.8	407.8	337.8
	PCB 167, 156, 157	359.9	289.9	361.9	291.8	371.9	301.9	373.9	303.8
	PCB-138, 101, 153	359.9	289.9	361.9	291.8	371.9	301.9	373.9	303.8
NDL PCBs	**PCB-52**	289.9	219.9	291.9	221.9	301.9	231.9	303.9	233.9
	PCB-28	256.0	186.0	258.0	188.0	268.0	198.0	270.0	200.0
	PCB-180	393.8	323.8	395.8	325.8	405.8	335.8	407.8	337.8
Recovery Standards dioxin	**1278-TCDF**	NA				315.9	251.9	317.9	253.9
	123469-HxCDF					385.8	321.9	387.8	323.9
	1234689-HpCDF					419.8	355.8	421.8	357.8

Recovery Standards PCBs	**PCB 70**	NA	301.9	231.9	303.9	233.9
	PCB 127		339.9	269.9	337.9	267.9
	PCB 170		405.8	335.8	407.8	337.8



The selection of precursor 1 and precursor 2 with 2 Da difference is based on the natural abundance of ^{35}Cl: ^{37}Cl = 3:1

Fig 2.10 Fragmentation pattern of 2,3,7,8- TCDD

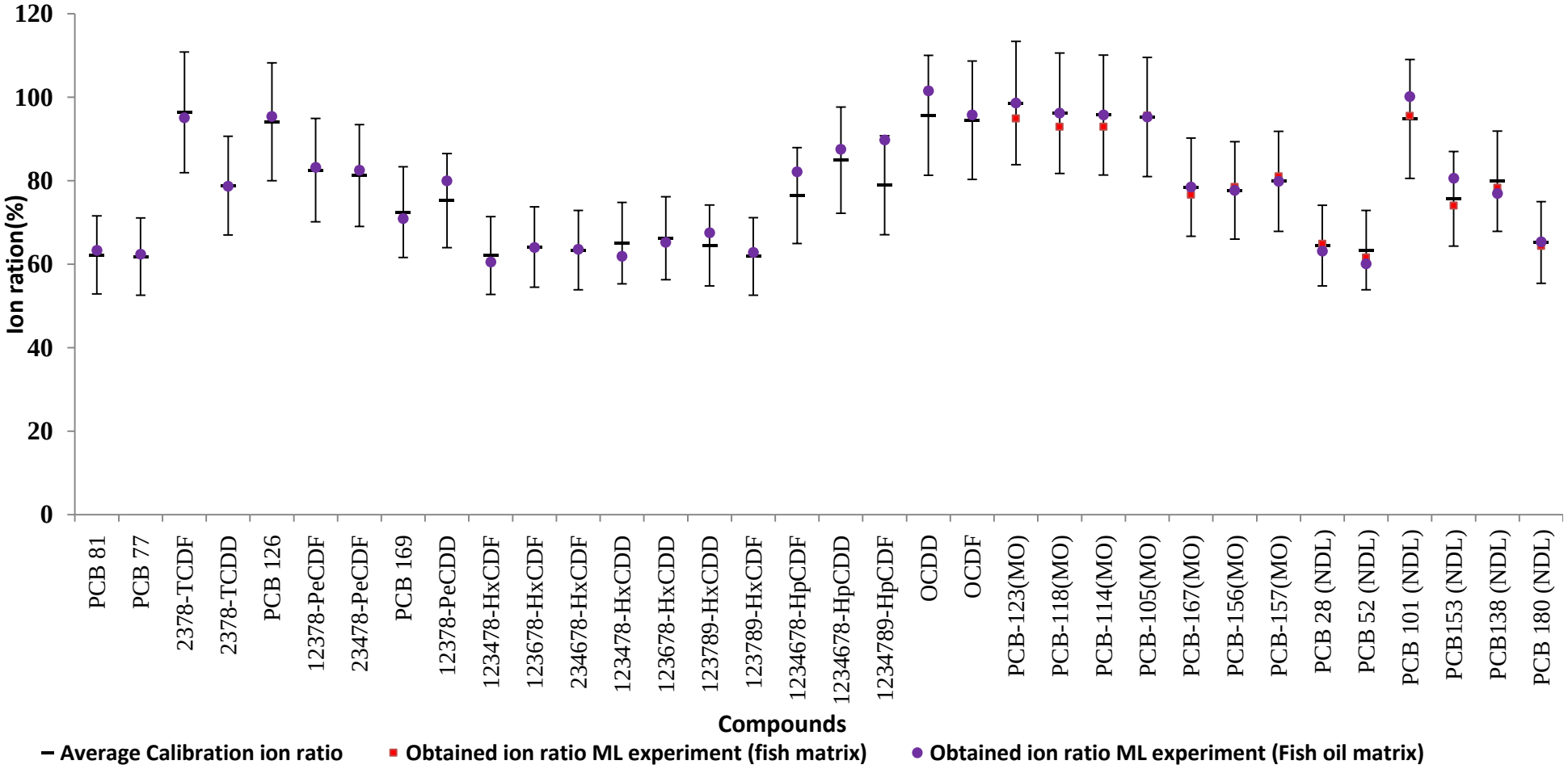


Fig 2.11: Obtained ion ratio (Average calibration, fish and fish oil ML experiment), error bars represents ± 15 % allowed tolerance as per EU regulation 644/ 2017

2.4.7. Estimation of measurement uncertainty

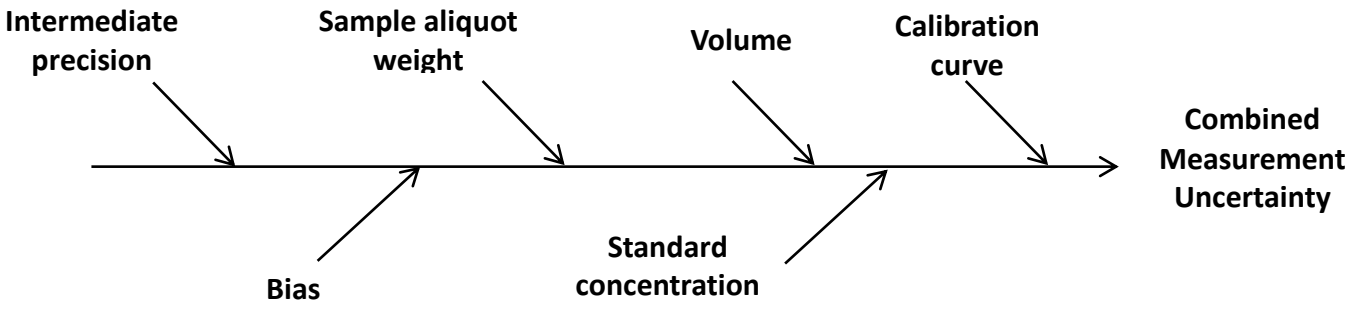


Fig 2.12 Measurement uncertainty contributions to combined uncertainty

The estimation of matrix-specific measurement uncertainty in laboratories conducting PCDD/F and PCB analysis using Isotope Dilution Mass Spectrometry is guided by the "Guidance Document on Measurement Uncertainty for Laboratories performing PCDD/F and PCB Analysis using Isotope Dilution Mass Spectrometry" by EU. This comprehensive document outlines three distinct approaches for uncertainty estimation: the empirical or top-down approach, theoretical or bottom-up approach, and semi-empirical approach. Each approach offers its unique methodology tailored to the laboratory's expertise, available data, and the nature of the analyses being conducted. The empirical approach, commonly referred to as the top-down approach, involves leveraging empirical data obtained from repeated measurements or proficiency testing. By observing variations in actual measurement results, this approach quantifies uncertainty by working downwards from the observed data. While this method provides a practical means of estimating uncertainty based on real-world measurements, it may be limited by the availability of sufficient empirical data, especially for laboratories new to the analysis. Conversely, the theoretical approach, also known as the bottom-up approach, starts from fundamental principles to identify and quantify potential sources of uncertainty in the measurement process. By systematically considering each contributing factor and its associated uncertainty, this approach aggregates individual uncertainties to estimate the overall uncertainty of the measurement. While

theoretically rigorous, this method requires a deep understanding of the measurement process and may rely on assumptions that could introduce uncertainties themselves.

Given our laboratory's limited experience and participation in proficiency testing, we opted for the semi-empirical approach, which combines elements of both the empirical and theoretical approaches. This approach allows leveraging available empirical data while incorporating theoretical insights to estimate uncertainty. By utilizing equations provided in the guidance document and considering various uncertainty contributions (depicted as fish bone diagram, **Fig 2.12**), estimated measurement uncertainty effectively. The obtained results, as detailed in **Table 2.9**, demonstrate that the estimated uncertainty values for dioxins and ndl-PCBs were below the regulatory threshold of 38 %. This finding underscores the effectiveness of our developed methodology in ensuring measurement accuracy and compliance with regulatory requirements, despite our laboratory's relative novelty to the analysis.

Table 2.9

Estimated measurement uncertainty at ML leve of fish and fish oil matrices

SI No	Compounds	TEF	Fish matrix		Fish oil matrix	
			Expanded MU (±)	MU estimation at	Expanded MU (±)	MU estimation at
1	2378-TCDD	1	0.062	0.56	0.125	0.800
2	2378-TCDF	0.1	0.054	0.56	0.118	0.800
3	12378-PeCDD	1	0.205	1.40	0.216	2.00
4	12378-PeCDF	0.03	0.183	1.40	0.190	2.00
5	23478-PeCDF	0.3	0.199	1.40	0.314	2.00
6	123478-HxCDD	0.1	0.197	1.40	0.234	2.00
7	123678-HxCDF	0.1	0.136	1.40	0.250	2.00
8	123678-HxCDD	0.1	0.157	1.40	0.281	2.00
9	123678-HxCDF	0.1	0.215	1.40	0.312	2.00
10	123789-HxCDD	0.1	0.186	1.40	0.198	2.00
11	123789-HxCDF	0.1	0.127	1.40	0.309	2.00
12	234678-HxCDF	0.1	0.157	1.40	0.264	2.00
13	1234678-HpCDD	0.01	0.154	1.40	0.207	2.00
14	1234678-HpCDF	0.01	0.128	1.40	0.199	2.00
15	1234789-HpCDF	0.01	0.211	1.40	0.234	2.00
16	OCDF	0.0003	0.269	2.80	0.637	4.00
17	OCDD	0.0003	0.410	2.80	0.396	4.00
18	PCB 77	0.0001	2.64	22.96	12.8	114.8
19	PCB 81	0.0003	2.57	22.96	12.2	114.8
20	PCB 126	0.1	3.07	22.96	11.9	114.8
21	PCB 169	0.03	2.81	22.96	13.9	114.8
22	PCB-123(MO)	0.00003	2.68	22.96	10.4	114.8
23	PCB-118(MO)	0.00003	3.10	22.96	14.8	114.8
24	PCB-114(MO)	0.00003	2.63	22.96	15.5	114.8
25	PCB-105(MO)	0.00003	3.58	22.96	14.5	114.8
26	PCB-167(MO)	0.00003	2.75	22.96	16.4	114.8
27	PCB-156(MO)	0.00003	2.16	22.96	11.1	114.8
28	PCB-157(MO)	0.00003	3.20	22.96	10.9	114.8
29	PCB-189(MO)	0.00003	3.56	22.96	15.3	114.8
∑ PCDD/Fs –dl PCBs TEQ			**0.85**	**6.50**	**2.3**	**20.0**
% uncertainty			**13.07 %**		**11.5%**	
30	PCB-28(ind)	NA	416.8	12500.0	1370.2	29166.7
31	PCB-52(ind)		331.1	12500.0	1076.3	29166.7
32	PCB-101(ind)		377.1	12500.0	647.1	29166.7
33	PCB-153(ind)		428.4	12500.0	798.3	29166.7
34	PCB-138(ind)		386.5	12500.0	1322.4	29166.7
35	PCB-180(ind)		310.3	12500.0	911.1	29166.7

∑ NDL-PCBs	**2250.2**	**75000.0**	**6125.4**	**175000.0**
% Uncertianty	**3.00%**		**3.5%**	

Units: Fish matrix (pg/g wet weight), Fish oil matrix pg/g, relative to a feed with 12 % moisture content

2.4.8. Participation in proficiency test

The laboratory, utilizing the developed analytical methodology, participated in an international proficiency test program administered by FAPAS^{TM}, focusing on the fish matrix. The lyophilized PT sample received underwent processing using optimized extraction and sample cleanup techniques. The Z-Score serves as the performance indicator for participant laboratories in proficiency testing. This statistical tool is employed to assess participant results, identify outliers, and exclude their data from the proficiency test outcome. A Z-Score value between +2 and -2 is deemed satisfactory. The results of Z-Scores, both group-wise and congener-wise, obtained in the PT program are presented in **Table 2.10** and **Fig. 2.13**, respectively. The obtained Z-Scores were ≤ 2 for 95% individual congeners and group-wise analytes, highlights the efficacy of the developed method in conducting bias-free quantification of analytes at an ultra-trace level.

Table 2.10
Congener group wise obtained z-scores

Category	Lab value	Assigned value	Z score value
	ng/kg		
PCDD/Fs TEQ (UB)	5.69	6.47	0.6
PCBs TEQ (UB)	2.42	3	-0.9
PCDD/Fs -PCBs TEQ (UB)	9.81	8.83	0.5

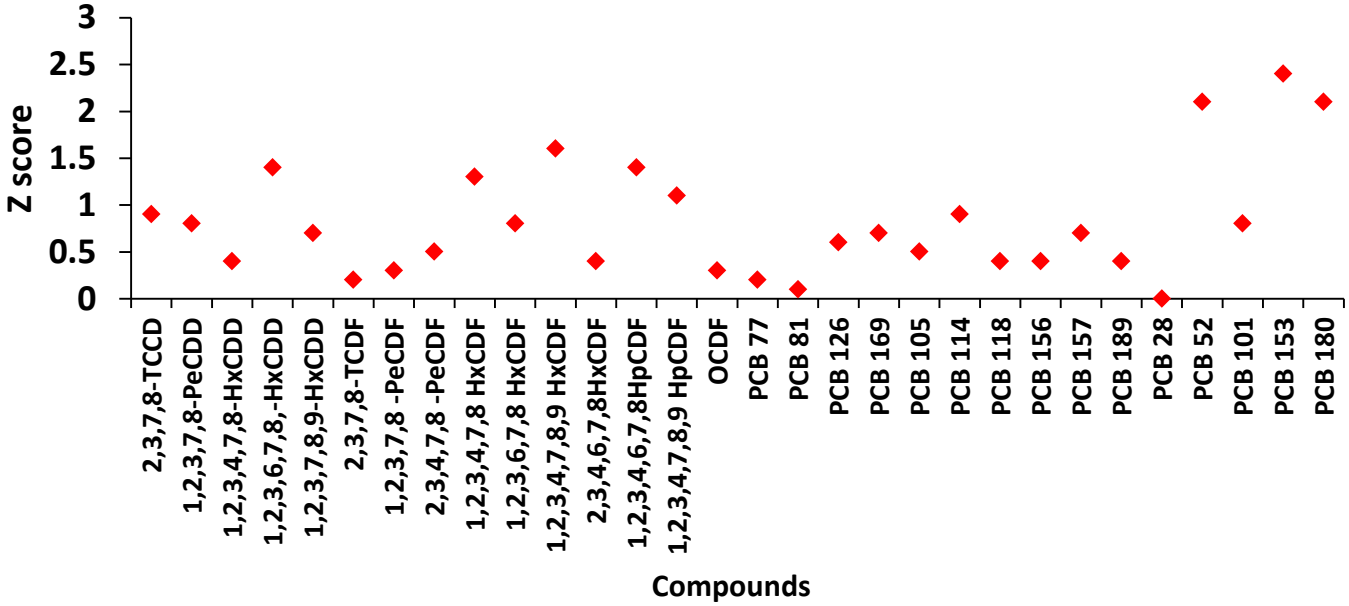


Fig 2.13 Congener wise obtained z-score values

2.5. Conclusion

This chapter focuses on the critical validation of the developed GC-MS/MS methodology in two pivotal matrices: fish and fish oil. The optimization process involved the refinement of Soxhlet and PLE-based extraction methods, as well as multilayer silica, activated carbon, and alumina-based column chromatography for sample cleanup, ensuring internal standard recovery rates within the optimal range of 70-110%. Each criterion outlined in the regulation was thoroughly assessed and found to meet satisfactory standards. Central to the validation procedure were spike recovery experiments conducted at ML, ML/2, and 2ML levels, yielding precision and accuracy values within acceptable ranges for both matrices. The estimated measurement uncertainty values for PCDD/Fs & dl-PCBs (13.07% in fish matrix and 11.5% in fish oil matrix) and ndl PCBs (3.00% in fish matrix and 3.5% in fish oil matrix) were found to be satisfactory compared to the regulatory limit of 38%. Additionally, the obtained z scores between ± 2 in the fish matrix proficiency testing, conducted using the developed methodology, further validate its efficacy. In summary, this research establishes a critical validation procedure for GC-MS/MS-based quantitation of dioxins and PCBs, which can be extrapolated to different matrices. By ensuring unbiased and cost-effective quantitation of these contaminants, this methodology holds significant implications for enhancing food and feed safety measures.

Chapter

3

Development of a field deployable analytical workflow to assess the current state of a historical POPs hotspot in India

3.1. Abstract

This chapter introduces a comprehensive workflow aimed at assessing the environmental and health risks associated with dioxin-like Persistent Organic Pollutants (dl-POPs) in industrial hotspot regions. There is a critical need for reliable, cost-effective, and easy-to-use analytical methods that can be deployed in the field for routine monitoring of dl-POPs, especially in developing nations. This study addresses the lacunae by using the developed and validated gas chromatography triple quadruple mass spectrometer based analytical methodology (**chapter 2**) as per the European Union regulation 644/2017, substituting conventional magnetic sector high resolution mass spectrometer technique. To validate the effectiveness of this methodology in real-world scenarios, fish and sediment samples were collected from the Eloor-Edayar industrial belt, a significant dl-POPs hotspot in India. Analysis of congener profiles suggests that dl-POPs primarily originate from precursor pathways, indicating potential release of chlorinated precursor species from surrounding industrial activities. Compared to control sites, fish samples from these hotspots exhibited significantly higher levels of polychlorinated dibenzo-p-dioxin/furans (PCDD/Fs) and polychlorinated biphenyls (PCBs), with increases of 8 and 30 times, respectively. A robust positive correlation ($p < 0.05$) was found between dl-POPs levels in fish and sediment samples, and Biota sediment accumulation factors for PCDD/Fs and dl-PCBs ranged from 0.019 to 0.092 and 0.004 to 0.671, respectively. The estimated weekly intake of dl-POPs from fish consumption in the study area exceeded the maximum levels recommended by the European Food Safety Authority by 3 to 24 times. Therefore, it is crucial to conduct regular monitoring of dl-POPs using user-friendly and validated confirmatory tools to ensure the protection of human health and the environment.

3.2. Introduction

In today's world, environmental contaminants pose significant threats to food safety and human health. Among the most hazardous are Polychlorinated dibenzo-dioxin/furans (PCDD/Fs) (17 congeners) and Polychlorinated biphenyls (PCBs) (18 congeners), recognized for their Persistent, Bio accumulative, Toxic, and Long-Range Transport (PBT/LRT) characteristics and adverse health effects at ultra-trace levels (Lauby-Secretan et al., 2013; Matthies et al., 2016) . These compounds were initially listed among the twelve Persistent Organic Pollutants (POPs) under the Stockholm Convention due to their extreme toxicity and associated health risks, including

chloracne, reproductive dysfunction, immune suppression, and cancer (Bertazzi et al., 2001; Kogevinas et al., 2001; White and Birnbaum, 2009). Consequently, regular surveillance of dioxin-like (dl)-POPs, particularly in hotspot or contaminated areas, is strongly recommended. As part of global POPs monitoring efforts, the International Pollutant Elimination Network (IPEN) compiled a "World map of POPs waste hotspots," identifying forty such locations worldwide, with many situated in the least developed or developing economies (Watson et al., 2009).

Previous studies have largely overlooked the monitoring of dl-POPs in industrial hotspot regions, with a predominant focus on dl-POPs hotspots in Vietnam due to the predominant global attention towards Agent Orange contamination (Van Thoung et al., 2015; Hoang et al., 2014). Despite routine surveillance in developed countries, developing and transitioning economies lack validated analytical methods, facing significant capital costs (ranging from 500 to 600 K USD) and operational expenses associated with confirmatory analysis via High Resolution Gas Chromatograph-High Resolution Mass Spectrometer (HRGC-sector HRMS) (Palmiotto et al., 2013). Advancements in mass spectrometric techniques, particularly GC coupled with triple quadrupole mass spectrometer MS (GC-MS/MS), offer a promising alternative. GC-MS/MS provides comparable results to HRMS, with advantages such as lower installation costs (ranging from 150 to 200 K USD) and reduced operational and maintenance needs. Recognizing this, international bodies like the European Union (EU) and the United States Environmental Protection Agency (USEPA) have established performance-based criteria for validating GC-MS/MS for confirmatory analysis of dioxins and PCBs (USEPA, 2022; European Union, 2017). While studies have validated GC-MS/MS methods for specific food and feed matrices, its broader application in surveillance efforts targeting dl-POPs hotspots remains limited Ábalos et al., 2016; Franchina et al., 2019; L'Homme et al., 2015**)**. There is a significant opportunity to leverage GC-MS/MS as a cost-effective and reliable method for monitoring dl-POPs in hotspot regions, facilitating a more comprehensive assessment of environmental and health risks.

The primary objective of this chapter is to use the developed GC-MS/MS methodology for analyzing dl-POPs (in chapter 2) in hotspot regions. Field implementation studies were conducted in the Eloor-Edayar region, the sole POPs hotspot in India among the 40 identified globally. This area raises particular concern due to its history of dichlorodiphenyltrichloroethane

(DDT) and other organochlorine pesticide manufacturing plants, which could lead to the unintentional formation of dl-POPs (Divya et al., 2021). Notably, The Hindustan Insecticides Ltd (HIL) operated a DDT manufacturing unit until 2018, making it the sole global producer of DDT. The well-documented unintentional generation of dl-POPs during chlorinated pesticides manufacturing processes adds to the region's significance (Liebens and Mohrherr, 2015). Despite this known risk, dl-POPs in the Eloor-Edayar region, like many other global hotspots, have been inadequately monitored due to the complexity and high cost associated with confirmatory analysis (Stefanovic et al., 2019).

The study extensively examines the congener fingerprints, group-wise abundance of congeners, and correlation patterns of dl-POPs in fish and sediment samples. It also evaluates the Biota Sediment Accumulation Factor (BSAF) values to comprehend the extent of contaminant transfer into the food chain. Additionally, the research aims to assess the health risks for fish consumers in the region by estimating daily intake and comparing it against tolerable levels set by global stakeholders such as the World Health Organization (WHO) and European Food Safety Authority (EFSA). Consequently, this study has the potential to develop into a systematic and cost-effective analytical workflow for the global monitoring of dl-POPs in contaminated sites.

3.3. Materials and methods

3.3.1. Development of analytical workflow for the selected study site

The study area, the Eloor-Edayar industrial POPs hotspot, covers approximately 11.2 km^2 and is situated between 76.29250°–76.30880° E (longitude) and 10.08100°-10.07720° N (latitude). It stands as the sole Comprehensive Environmental Performance Indexed (CEPI) area in Kerala, a southern state in India, hosting over 247 small and medium-sized industries spanning chemical, petrochemical, pesticide, leather, and fertilizer manufacturing sectors. To develop the analytical workflow, an initial comprehensive background study was undertaken to identify potential contamination sources, review past investigations, and gather baseline data from stakeholders. Subsequently, sediments and fish samples were chosen as primary markers to assess the enviro-food-health risks associated with dl-POPs in riverine ecosystems. Notably, the Periyar River has witnessed a decline in fish species from 35 in 1980 to 12 due to severe industrial pollution and water quality deterioration (Ambily and Menon, 2019; Ramachandra et al., 2012). For comparative purposes, fish samples were also collected from Vellayani Lake in

Thiruvananthapuram, Kerala, a non-industrial area known for relatively low pollution levels (Das et al., 2019), and from the open market as a market control sample. The developed GC-MS/MS based analytical methodology (**chapter 2**) was applied to assess dl-POPs levels in samples from the aforementioned sites and analyzed using statistical and bio-monitoring tools to elucidate critical parameters representing the hotspot's current status.

3.3.2. Sampling

In this study, a total of 72 fish samples (three samples per species per sampling site) and 18 sediment samples (three samples per sampling site) were gathered from the river surrounding the hotspot. Six specific locations were identified in the river where local fishermen frequently engage in fishing activities to supply fish for the community, all situated around the industrial area (**Fig 3.1**). These sampling points were carefully selected to assess health risks to fish consumers without bias. Various characteristics were considered in selecting the sampling sites, including their proximity to industrial zones, canals connecting industrial areas, and the river's main flow. Out of the six sampling points, four (SP1, SP3, SP4, and SP5) were located in the main flow of the Periyar River, while the remaining two (SP2 and SP6) were from its distributary canal. Four fish species—Etroplus suratensis (E. suratensis), Mugil Cephalus (M. cephalus), Horabagrus brachysoma (H. brachysoma), and Oreochromis mossambicus (O. mossambicus)—were selected for the study based on their importance in the local diet and their abundance in the area. Fish samples were collected from each sampling point and control sites using cast net catching methods, while market control samples were obtained from the open market. Comparable-sized and weighted fish samples were chosen for analysis to determine species-specific contaminant levels at each sampling point. Corresponding sediment samples were also collected from the same locations where fish were sampled using a grab sampler. Analyzing the trend in dl-POPs concentration between the hotspot region, control site, and general market could shed light on the role of different pollution sources. All samples were stored at -20°C until analysis in the laboratory.

3.3.3. Chemicals and consumables

All certified reference standards utilized for isotopic dilution calibration and confirmatory analysis, including native and labeled PCDD/Fs, PCBs, and Dioxin/PCBs recovery/syringe

standards, were procured from Cambridge Isotopic Laboratories (CIL), based in Massachusetts, USA. Labeled non-ortho and mono-ortho PCBs (NO and MO PCBs) were sourced from Wellington Laboratories in Guelph, Canada. Headspace GC grade solvents were obtained from Spectrochem in India, while high-purity n-nonane keeper solvent was acquired from E-Merck in Germany. Multilayer silica, alumina, and reversible carbon columns used for sample purification were obtained from Fluid Management Systems (FMS Inc.) located in Waltham, MA, USA. Ultra-high purity helium and nitrogen gases (99.999%, sourced from Bhoruka in India) served as carrier and collision gases, respectively.

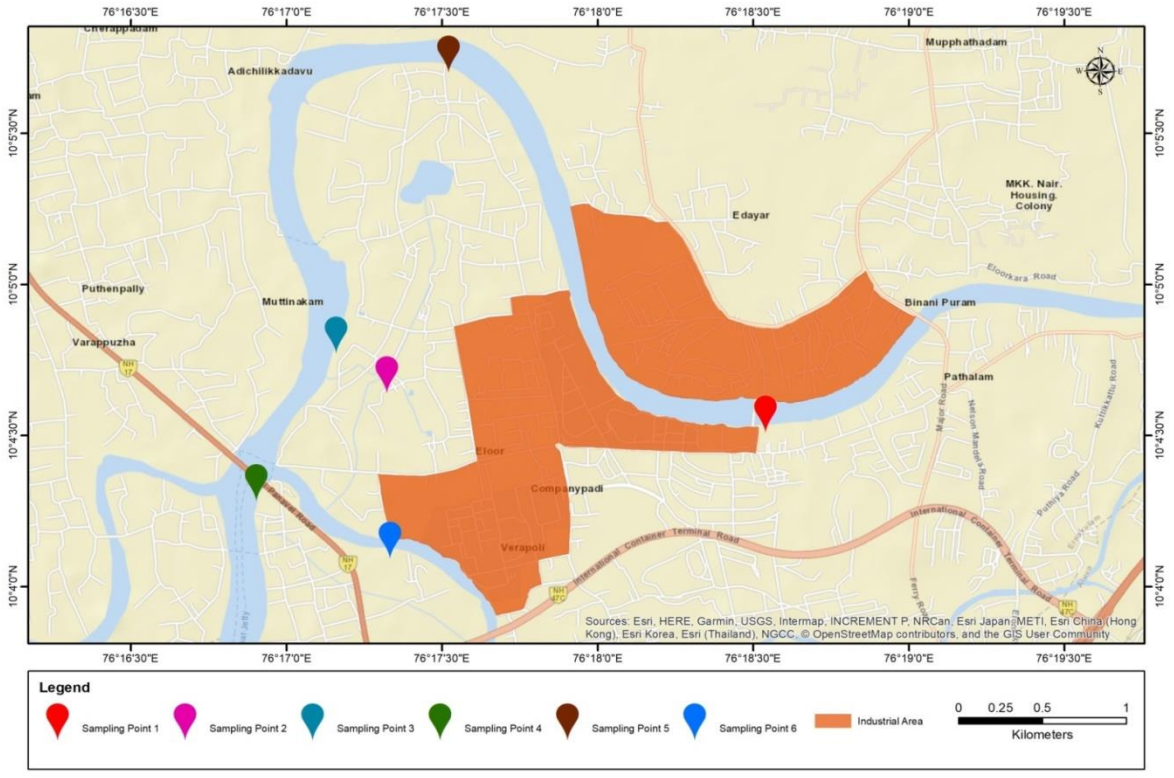


Fig.3.1. Map showing study area with sampling points

3.3.4. Sample preparation and quantification of dioxins and PCBs

Sample preparation and quantification for dl-POPs in both fish and sediment samples were conducted at the ISO/IEC 17025:2017 accredited Dioxin Research Laboratory (DRL) within CSIR-NIIST. Sediment samples underwent air-drying, and Total Organic Carbon (TOC) analysis was performed using a TOC analyzer (3100 multi N/C, Analytikjena, Germany) equipped with

an HT 1300 solid module. Homogenized tissue samples were freeze-dried, while sediment samples were ground into powder before extraction. In typical experiments, 10 g of tissue and sediment samples were taken on wet and dry weight bases, respectively. Samples were combined with anhydrous sodium sulfate and spiked with ^{13}C labeled internal standards for all PCDD/F and PCB congeners before undergoing extraction using a customized Accelerated Solvent Extraction (ASE) system with a neutral silica layer-assisted inline cleanup step. Lipid content in fish samples was determined following EC152/2009 guidelines (EU 152/2009, 2009). The sample cleanup process was performed using the semi-automated EZprep 123 workstation (FMS Inc., USA), comprising a three-column setup with multi-layer silica, activated carbon, and alumina connected in series. The concentrated extract from ASE, dissolved in hexane solvent, was introduced into the cleanup system after conditioning. The resulting two fractions (Fraction 1 for PCDD/Fs & NO-PCBs and Fraction 2 for MO & non-dioxin-like (ndl)-PCBs) underwent nitrogen evaporation concentration (Supervap, FMS Inc, USA) and were reconstituted with n-nonane to 200 μL after spiking with a recovery standard. Both fractions were then quantified using a custom-validated GC-MS/MS system for dioxins & PCB analysis. Quantification of Fraction 1 and Fraction 2 was carried out separately using an Agilent GC-MS/MS system, which included a 7890 B gas chromatograph equipped with a 7690 A automatic liquid sampler and a 7000C triple quadrupole mass spectrometer. Effective congener separation was achieved using a classic DB-5 MS 60 m × 250 μm × 0.25 μm (Agilent) capillary column. Instrument calibration and auto-tuning were performed biweekly. Optimized extraction, cleanup, and quantification parameters are detailed in **Table 3.1**.

3.3.5. Estimation of bioaccumulation trend and health risk

The Biota Sediment Accumulation Factor (BSAF) serves as a primary screening tool employed by regulatory bodies globally to evaluate the risk posed to aquatic organisms by organic pollutants and to devise remediation strategies (Burkhard et al., 2004). BSAF values were estimated using fat-normalized contaminant levels in fish tissue and TOC-normalized sediment levels, which were specific to fish species and sampling points (**Eq 3.1**). Regression analysis of the data was conducted using JASP statistical software to determine statistically significant Pearson's correlation coefficients, with a significance level set at $p < 0.05$ (**Eq 3.2**). Assessment of the health risks by estimating daily intake (**Eq 3.3**) posed by these contaminants to fish

consumers in the study area, compared to controls (field and market), was based on observed levels in various fish species and the fish consumption pattern outlined by the National Institute of Nutrition, India (NIN, 2020). This assessment was further compared with the Tolerable Weekly Intake (TWI) recommended by the European Union to gauge the pollution's impact (EFSA CONTAM Panel, 2018).

$$\textbf{Biota Sediment Accumulation Factor, } BSAF = \frac{C_{fish}}{C_{sediment}} \text{.....(Eq 3. 1)}$$

(C_{fish} is the fat normalized contaminant level in fish tissue in pgTEQ/g fat and $C_{sediment}$ is the total organic carbon normalized contaminant level in the sediment in pgTEQ/g total organic carbon)

$$\textbf{Pearson's Correlation coefficient, } r = \frac{\sum(x_i-\bar{x})(y-\bar{y})}{\sqrt{\sum(x_i-\bar{x})^2(y-\bar{y})^2}} \text{.....(Eq 3.2)}$$

x_i = dioxin or PCB levels in sediment

$\bar{x}$ = Mean value of dioxin or PCBs levels in sediment

y_i = Mean value of dioxin or PCBs levels in fish

$\bar{y}$ = Mean value of dioxin or PCBs levels in fish

$$\textbf{Daily intake} = \frac{\textbf{Combined dioxins \& DL-PCBs level } \left(\frac{pg}{g}TEQ\right) \times \textbf{Daily fish consumption } (g)}{\textbf{Average adult body weight } (Kg)} \text{.....(Eq 3.3)}$$

Table 3.1
Analytical method overview

Sample Preparation/ Pretreatment			**Quantification of analytes**	
Sample Extraction			Instrument: Gas chromatograph coupled with tandem in space triple quadruple mass spectrometer	
Equipment: Automated Pressurized Liquid Extraction System: Dionex ASE-350, Thermofisher Scientific			Gas chromatograph System: 7890 GC & 7690 A Automatic Liquid Sampler, Agilent Technologies Triple quadruple mass spectrometer: 7000C, Agilent Technologies	
Optimized analytical method			**Optimized analytical method- Gas chromatograph**	
Parameters	Fish tissue samples	Sediment samples	Capillary column specification	Agilent DB-5 MS, 60 m × 0.25 mm, 0.1 µm
Extraction solvent	Hexane: DCM (1:1)	Toluene	Capillary column pneumatics	Constant flow (0.98 mL/min, He carrier gas)
Pressure	1700 psi	1700 psi	Liner	5190-2293 Ultra-Inert, single taper
Oven temperature	130 ^{o}C	180^{o}C	Injection mode	Solvent vent mode
Static time	5 min	10 min	Injection volume	4 µL
Static cycles	3	4	Inlet Temperature program	120^{0}C for 0.08 min, 100^{0}C/min to 160^{0}C, hold 0.15 min, 700^{0}C/min to 330^{0}C, hold 8 min, 10^{0}C/min to 250^{0}C
Rinse volume	80%	80%		
Nitrogen purge time	5 min	5 min		
Sample Clean up			Oven temperature program	60^{0}C for 1 min, 30^{0}C/min to 270^{0}C, hold 1 min, 2^{0}C/min to 310^{0}C, 10^{0}C/min to 325^{0}C, hold 5 min
Analytical technique used: Semi automated multilayer silica, alumina and carbon based column chromatography System: EZprep-123, Fluid Management Systems (FMS)			**Optimized analytical method- MS-MS**	
Optimized analytical method			Ionization mode	EI 70 eV
Chromatographic column configuration: Multilayer silica-carbon -alumina in series			Acquisition mode	MRM (Two precursors are two specific product ions)
Column conditioning	40 ml Hexane		Interface temperature	300^{0}C
Sample loading	30 ml (In Hexane)		Ion source temperature	330^{0}C
Elution volume	130 m l Hexane		Solvent delay	11 min
Carbon reverse elution	40 mlToluene (Fraction 1: PCDD/Fs & NO PCBs) (Fraction 2: MO & NDL)		Quadruple temperature	Q1 (150^{0}C) Q2 (150^{0}C)

3.4. Results and discussions

3.4.1. GC-MS/MS methodology vs GC- HR MS

The comparative analysis in Chapter 2 illustrates that the developed GC-MS/MS methodology offers similar effective performance to GC-HRMS in fulfilling specific regulatory requirements for confirmatory analysis of dl-POPs. Despite employing different mass spectrometric quantification strategies (Mass accuracy vis-a-vis MRM induced selectivity), both technologies achieve comparable results. HR-MS utilizes ion acceleration and a magnetic field for differentiation, yielding superior mass resolution (up to four decimal points) via Selective Ion Monitoring (SIM). Conversely, GC-MS/MS leverages multi-stage mass spectrometric filtering through Multiple Reaction Monitoring (MRM), ensuring exceptional selectivity in quantitation. Regarding technical specifications, HRMS targets a resolving power of 10,000 (with a 10% valley) at m/z 304.9824 (PFK), while MS/MS maintains a resolution of 0.7 amu/Da for effective dl-POPs analysis. Both techniques utilize ion ratio ±15% windows, with HRMS focusing on molecular fragment ratios (quantifier ion to qualifier confirmatory ion) following USEPA 1613 B standards, and MS/MS concentrating on precursor ion to product ion ratios as per USEPA 1613 B (USEPA 1613B, 1997) guidelines. These ion ratio measurements serve as quality control measures in both methodologies, with the validated GC-MS/MS method consistently meeting these stringent criteria. Efficient sample clean-up is essential to meet regulatory standards and ensure unbiased results, despite any relaxation in mass resolution. Furthermore, advancements in ion source efficiency in MS/MS systems can enhance sensitivity, enabling reduced sample size, mitigating matrix effects, and decreasing sample preparation time, ultimately extending the lifespan of consumables. However, HRMS entails higher installation and maintenance costs, lacks stability, requires time-consuming maintenance, and demands highly trained professionals for operation and upkeep. The detailed comparison of these two technologies is provided in **Table 3.2**.

Table 3.2
Comparison of HRGC-HRMS Vs GC-MS/MS

Sl. No.	Attributes	HRGC-HRMS	GC-MS/MS
		Detection of the precursor ions within a narrow mass window of 100 ppm	Two times mass filtering
1	Detection technique	Works on SIM mode	Works on MRM mode
		Mass accuracy up to 5 points after decimal.	2 MRM transitions monitored per analyte and labelled compounds
2	Ionisation mode	EI	EI
3	Electron energy	30-50 ev	70 eV
4	Quantification	Yes	Yes
5	Use of ISTD	Yes	Yes
6	Mass locking	Tuning with PFA/PFTBA solution	Tuning with PFTBA solution
7	Source suppression check	Yes	Yes
8	Interference by chlorodiphenyl ethers established and monitored	Yes	Yes
9	Resolution	10000 (10% valley)	0.7 amu/Da
10	GC Column	60 m DB-5 MS	60 m DB-5 MS
11	Capital investment cost	350-400 Lakhs	100-150 Lakhs
12	Operating costs	High	Medium
13	Skill and training of analyst	High	Medium

3.4.2. Trends of PCDD/Fs and PCBs in sediment samples

The levels of PCDD/Fs in sediment samples exhibited a range from 6.78 to 35.6 pgTEQ/g dry weight (d.w.) across various sampling sites. Notably, the most toxic congener, 2378-TCDD, consistently made the largest contribution to the total TEQ of PCDD/Fs at all six sampling points, followed by 2378-TCDF and 123789-HxCDD. However, the absolute concentration of OCDD displayed higher values in all sediment samples, ranging from 194 to 12114 pg/g dry weight. This observation aligns with congener fingerprint data from multiple studies, often

referred to as the "OCDD anomaly" (Kim et al., 2009; Nieuwoudt et al., 2009; Ren et al., 2009; Ssebugere et al., 2013). While the exact mechanism remains unclear, the prevalence of poor combustion conditions and higher chlorine content tends to favor the formation of kinetically feasible higher chlorinated congeners such as OCDD (Stanmore, 2004). Additionally, the poor water solubility of OCDD and its affinity to fine particles may lead to long-term accumulation, especially in organic-rich sediment compared to lower chlorinated congeners (Nunes et al., 2014; Van Thuong et al., 2015). Ratios of PCDFs to PCDDs calculated in most sediment samples were <1, indicating that the formation pathway likely involves precursor routes rather than de novo synthesis (chapter 1, 1.2.2)(Huang and Buekens, 1995). There's a high likelihood of emissions of chlorinated precursor species from surrounding industrial areas that may contribute to or trigger the formation of PCDD/Fs (Divya et al., 2021). The calculated ΣPCDD/Fs for sediment samples in this study were higher compared to values reported in some major rivers worldwide, indicating the significant influence of critical industrial sources in the region (**Table 3.3**). Sampling points SP1 and SP6, closer to the industrial area (16–30 m), showed higher contamination levels than SP2 to SP4, which are relatively farther away from the industrial region (440–1150 m)

Table 3.3
Levels of PCDD/Fs concentrations obtained in sediment samples vis-à-vis previous studies

Sl No	River/Water body site description	Nation	Levels of dl-POPs (pgTEQ/g dry weight)				Reference
			∑PCDD/Fs		∑ dl-PCBs		
			Lowest	Highest	Lowest	Highest	
1	River Nile (Cairo region)	Egypt	1.8	38.1	0.08	1.3	(El-kady et al., 2007)
2	Han River	Korea	1.14	10.5	0.01	0.626	(Kim et al., 2009)
3	Wloclawek reservoir	Poland	4.69	51.23	-	-	(Niemirycz and Jankowska, 2011)
4	Vaal River Vanderbijlpark	Central South Africa	0.2	9.6	0.48	4.44	(Nieuwoudt et al., 2009)
5	Upper Scheldt	Belgium	1.43	12.0	0.79	4.05	(Sanctorum et al., 2011)
6	Industrial area Pearl river delta	China	3.71	14.7	-	-	(Zhang et al., 2009)
7	Kentucky lake	USA	2.0	44.0	-	-	(Loganathan et al., 2008)

8	Petrochemical hotspot, Pancevo	Serbia	23.0	100.0	6.1	7.7	(Kaisarevic et al., 2011)
9	Eloor –Edayar POPs hot spot	India	6.77	35.2	2.66	8.12	present study

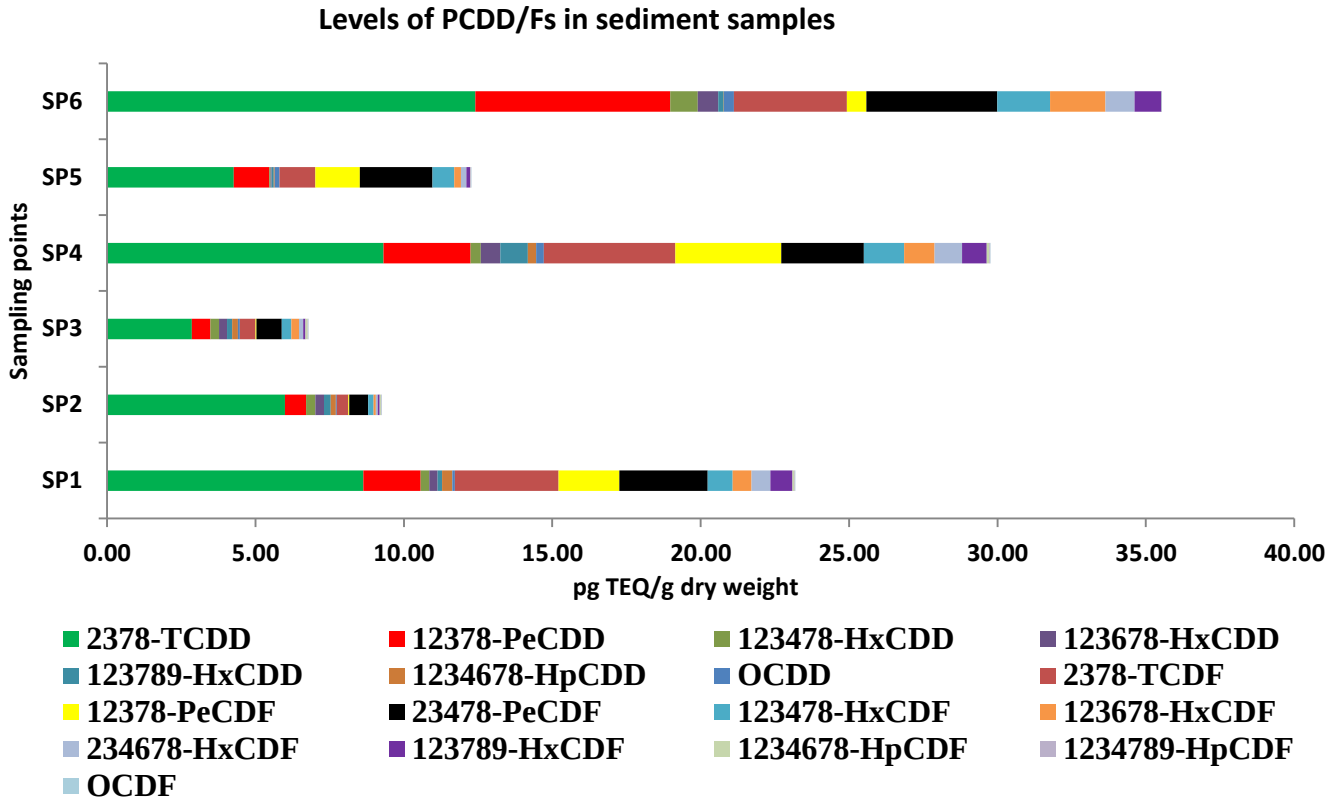
In the majority of samples analyzed, notably, the absolute concentrations of PCB 77 and PCB 81 were found to be high, consistent with reported values in the Nile and Han Rivers (El-kady et al., 2007; Kim et al., 2009). A notable positive correlation between levels of NO-PCBs and PCDD/Fs was observed at SP6 and SP4, suggesting increased emissions of dioxins and dl-PCBs in these regions from surrounding industrial activities. Furthermore, higher concentrations of higher chlorinated congeners (PCB 156, 167, 157, and 189) were observed in the analyzed samples due to their lower degradation potential in the environment and organisms (Lavandier et al., 2013). The combined toxic equivalence for dl-PCBs ranged from 2.66 to 8.12 pgTEQ/g dw in the sediment samples analyzed. This indicates a two-fold increase in dl-PCB concentrations compared to studies conducted elsewhere, such as in the Vaal River (South Africa) and Upper Scheldt River (Belgium-France) (Nieuwoudt et al., 2009; Sanctorum et al., 2011)..

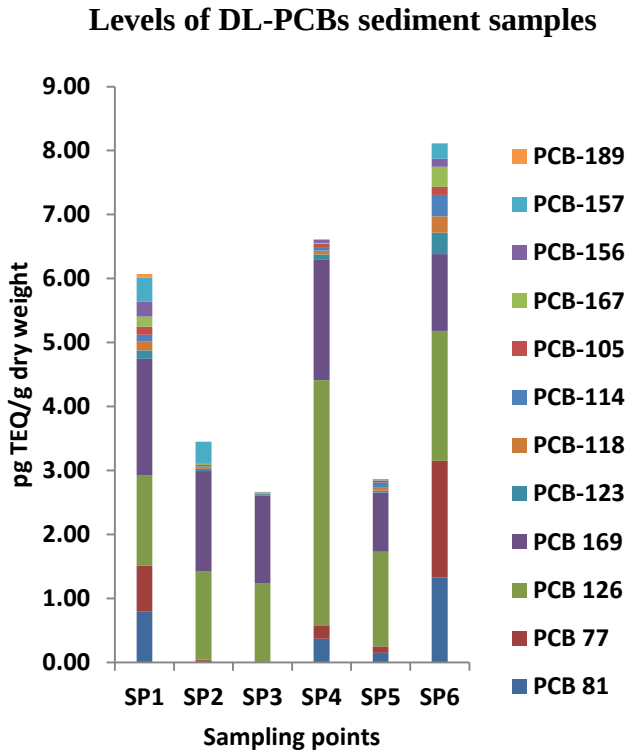
Similarly, an analogous trend in the abundance of more chlorinated congeners PCB 153, 138, and 180 towards the Σndl-PCBs concentration was observed. Sampling sites SP6 and SP4, situated near the industrial area (within 500 m), exhibited relatively higher values of Σndl-PCBs, comparable to levels reported in Lake Qarun of Egypt (Barakat et al., 2013; El-kady et al., 2007; Ssebugere et al., 2014). The individual concentrations of contaminants in the analyzed sediment samples are detailed in **Table 3.4**. Additionally, congener fingerprints of contaminants in the analyzed sediment samples and the percentage contribution of individual congeners to the cumulative TEQ are provided in **Fig. 3.2** and **3.3** respectively.

Table 3.4
Levels of PCDD/Fs and PCBs in the analyzed sediment samples

	List of Compounds	TEF	SP1	SP2	SP3	SP4	SP5	SP6
1	2378-TCDD	1	8.63	6.00	2.86	9.31	4.27	12.40
2	12378-PeCDD	1	1.94	0.71	0.62	2.94	1.19	6.57
3	123478-HxCDD	0.1	2.91	3.12	2.94	3.43	0.45	9.25
4	123678-HxCDD	0.1	2.83	2.86	2.60	6.54	0.36	6.96
5	123789-HxCDD	0.1	1.43	2.23	1.77	9.36	0.75	1.85
6	1234678-HpCDD	0.01	35.0	17.9	19.5	27.9	3.6	0.7
7	OCDD	0	300.8	94.9	195.1	856.0	510.7	1119.0
8	2378-TCDF	0.1	34.9	3.9	5.3	42.3	12.1	38.0
9	12378-PeCDF	0.03	68.0	1.2	1.3	118.9	50.2	22.0
10	23478-PeCDF	0.3	9.95	2.11	2.81	9.28	8.15	14.69
11	123478-HxCDF	0.1	8.32	1.84	3.32	13.60	7.30	17.83
12	123678-HxCDF	0.1	6.36	0.79	2.62	10.26	2.32	18.60
13	234678-HxCDF	0.1	6.46	0.63	1.40	9.24	1.75	9.83
14	123789-HxCDF	0.1	7.44	0.65	0.80	8.33	1.33	9.10
15	1234678-HpCDF	0.01	6.18	4.40	7.13	8.29	0.60	0.10
16	1234789-HpCDF	0.01	2.17	1.57	1.97	3.30	2.30	0.10
17	OCDF	0	2.87	1.38	5.52	2.00	0.40	0.43
∑ PCDDs (pg/g dry weight)			353.6	127.7	225.4	915.5	521.3	1156.7
∑ PCDFs (pg/g dry weight)			152.7	18.5	32.1	227.5	86.5	130.7
∑ PCDD/Fs(pg/g dry weight)			506.2	146.2	257.5	1143.0	607.7	1287.4
∑ PCDD/Fs (pg TEQ/g dry weight)			23.2	9.3	6.8	29.8	12.3	35.5
18	PCB 81	0.0003	2.36	0.07	0	0.7	0.34	6.09
19	PCB 77	0.0001	8.01	0.22	0.04	3.71	1.48	13.2
20	PCB 126	0.1	0.01	0.01	0.01	0.04	0.01	0.02
21	PCB 169	0.03	0.06	0.05	0.05	0.06	0.03	0.04
22	PCB-123	0.00003	4.44	1.08	0.65	2.81	1.07	11.2
23	PCB-118	0.00003	4.75	1.24	0.26	2.04	1.87	8.5
24	PCB-114	0.00003	3.37	0.32	0.11	1.81	2.47	11.1
25	PCB-105	0.00003	4.19	0.49	0.23	1.83	0.7	4.18
26	PCB-167	0.00003	5.37	0.46	0.22	0.45	0.27	10.6
27	PCB-156	0.00003	7.73	0.32	0.11	1.47	0.5	4.15
28	PCB-157	0.00003	12.5	11.2	0.17	0.2	0.04	7.86
29	PCB-189	0.00003	1.98	0.06	0.03	21	0.11	0.38
∑ NO PCBs (pg/g dry weight)			10.4	0.35	0.1	4.52	1.87	19.38
∑ MO PCBs (pg/g dry weight)			44.3	15.2	1.77	10.8	7.01	57.9
∑ dl PCBs(pg/g dry weight)			54.8	15.5	1.88	15.3	8.88	77.3

∑ dl PCBs (pg TEQ/g dry weight)			6.07	3.45	2.66	6.62	0.86	8.12
∑ PCDD/F/dl PCBs (pg TEQ/g dry weight)			29.3	12.7	9.4	36.4	15.1	43.7
30	PCB-28	NA	9.50	3.51	2.04	12.0	1.80	11.2
31	PCB-52		15.21	5.91	7.43	18.1	2.01	11.2
32	PCB-101		12.1	7.14	2.15	14.3	2.3	8.57
33	PCB-153		23.9	12.1	9.24	25.0	9.26	42.6
34	PCB-138		11.1	9.51	12.3	12.0	3.12	16.9
35	PCB-180		11.9	6.81	1.08	22.0	6.24	9.86
∑ NO PCBs (pg/g dry weight)			83.7	45.0	34.3	103.4	24.7	100.3





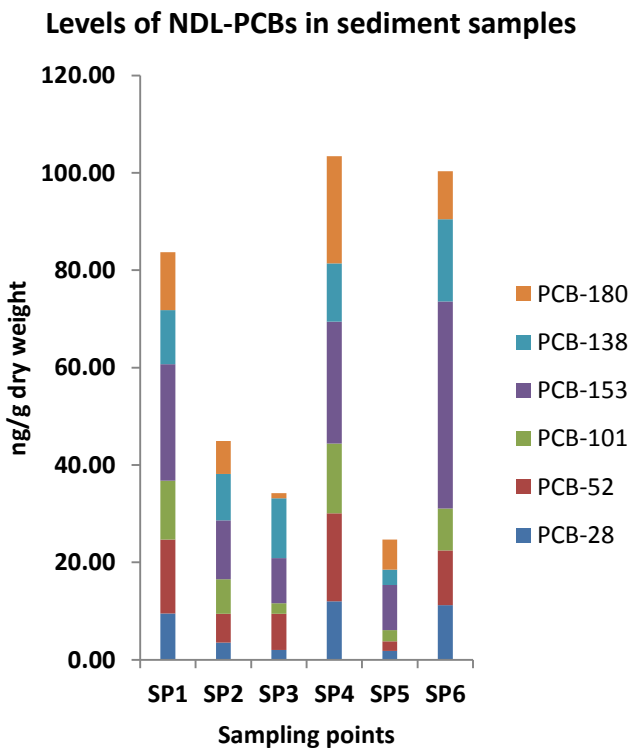


Fig. 3.2. Congener finger prints of contaminants in sediment samples

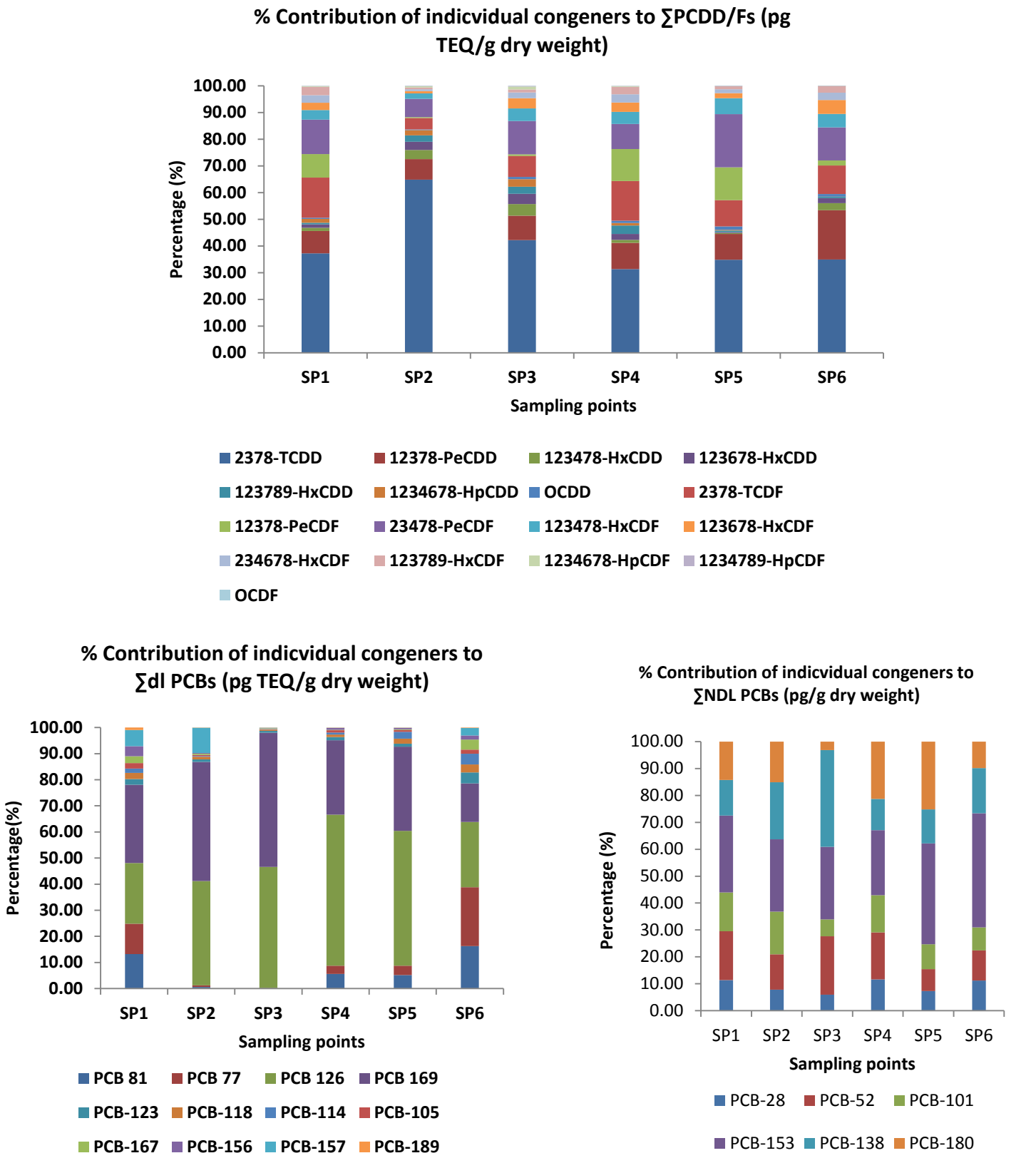


Fig. 3.3. Percentage composition of individual congeners to cumulative sum in sediment samples

3.4.3. Trends of PCDD/Fs and PCBs in sediment samples

The average fat content (w/w%) in the analyzed samples was as follows: E. suratensis (2.36 %), M. cephalus (5.06%), H. branchysoma (2.70%), and O. mossabicus (1.97%). Notably, higher levels were observed in E. suratensis and M. cephalus among the different fish species analyzed. The PCDD/Fs values obtained were higher compared to studies conducted in E. suratensis species from the coast of Sri Lanka, where the average Σ PCDD/Fs TEQ value was 0.19 pgTEQ/g, and in M. cephalus (0.18 pgTEQ/g) in northwest Florida respectively (Guruge and Tanabe, 2004; Karouna-Renier et al., 2011). Furthermore, the observed concentrations of PCDD/Fs in E. suratensis and M. cephalus at SP6 and SP4 approached or exceeded the EU regulation maximum level, i.e., 3.5 pg TEQ/g w. w. These locations are closer to the industrial belt, and the corresponding higher concentrations observed in sediment samples strongly indicate the transfer of dl-POPs from the sediment into fish tissue. Specifically, 2378 TCDD, 23478 PeCDF, and 12378 PeCDD congeners registered the largest contribution to the ΣPCDD/Fs TEQ in most analyzed samples of E. suratensis, while 2378-TCDF and 12378-PeCDD were predominant in M. cephalus. Regarding H. branchysoma, commonly referred to as yellow catfish, levels of PCDD/Fs were similar to those reported in several water bodies in southern Mississippi, USA, where the concentration varied between 0.09 and 3.14 pgTEQ/g. In most H. branchysoma samples analyzed, 2378-TCDD, 12378-PeCDD, and 23478-PeCDF congeners made the largest contribution to the total TEQ. Additionally, O. mossabicus species exhibited the lowest PCDD/Fs contamination, presumably due to their lower fat content. These discrepancies between intra/inter species are attributed to different dietary habits and trophic levels as reported elsewhere (Van der Oost et al., 2003). Notably, the benthic feeding behavior of M. cephalus showed a higher tendency to accumulate, leading to increased PCDD/Fs and dl-PCBs levels in most samples. However, the levels of dl-PCBs found in O. mossabicus and H. branchysoma were comparable, with small variations observed between different sampling sites, in contrast to the wide variation observed in E. suratensis and M. cephalus species. Bottom-dwelling fish species such as E. suratensis and M. cephalus sampled from SP6 exhibited higher Σ PCDD/Fs & dl-PCBs, indicating possible higher contamination with dl-POPs at SP6.

The higher chlorinated ndl-PCB congeners, such as 153, 138, and 180, dominated in all the examined fish samples, similar to the pattern observed in sediment samples owing to their low

degradation potency. The congener wise contaminant levels, congener fingerprints, and the percentage composition of the individual congeners towards the respective group-wise cumulative account are provided in **Table 3.5-3.8**, and **Fig. 3.4 to 3.7**, respectively. Significant interspecies differences in pollutant concentrations were observed even in samples taken from the same sampling point. These variations can be attributed to several factors, including differences in dietary habits, food intake, lipid content, and biochemical reactions in the organism (Barron, 1990; Ssebugere et al., 2013).. Additionally, species-specific differences in metabolism, detoxification mechanisms, and excretion processes can result in varying rates of pollutant absorption, metabolism, and elimination. However, these possible variations do not significantly affect the developed workflow, which is intended to study the extent of dl-POPs contamination and health risk estimations at the hotspot region. Control samples collected from both non-industrial areas and the open market exhibited lower levels compared to the hotspot, emphasizing the potential risk of POPs contamination in the study region due to industrial discharges and poor waste management. This contamination could pose long-term health risks to fish consumers.

Table 3.5
Levels of PCDD/Fs and PCBs in the analyzed fish sample *(E.suratensis)*

SI No	List of Compounds	*E.suratensis*					
		SP1	**SP2**	**SP3**	**SP4**	**SP5**	**SP6**
1	2378-TCDD	0.69	1.31	0.69	1.73	0.42	1.86
2	12378-PeCDD	0.42	<0.042	<0.042	<0.042	0.72	<0.042
3	123478-HxCDD	<0.04	<0.04	<0.04	<0.04	<0.04	1.65
4	123678-HxCDD	<0.033	0.14	<0.033	0.2	0.24	0.2
5	123789-HxCDD	<0.044	<0.044	<0.044	<0.044	0.81	<0.044
6	1234678-HpCDD	1.94	0.72	0.72	0.62	<0.198	0.62
7	OCDD	19.12	15.81	16	14.63	7.83	8.55
8	2378-TCDF	1.63	1.03	0.97	0.57	0.63	0.43
9	12378-PeCDF	7.41	0.43	0.5	10.8	7.52	14.12
10	23478-PeCDF	1.39	0.66	0.74	2.91	0.42	2.98
11	123478-HxCDF	0.99	<0.034	0.62	<0.034	0.2	<0.034
12	123678-HxCDF	0.24	0.1	0.21	<0.036	0.24	<0.036
13	234678-HxCDF	0.65	<0.028	0.66	<0.028	0.83	<0.028
14	123789-HxCDF	<0.032	<0.032	0.6	<0.032	0.13	<0.032
15	1234678-HpCDF	1.12	<0.043	2.8	<0.043	<0.043	<0.043
16	1234789-HpCDF	<0.031	<0.031	0.41	<0.031	0.26	<0.031

17	OCDF	0.13	0.2	0.15	0.13	<0.052	0.13
∑ PCDDs (pg/g wet weight)		22.2	18.0	17.4	17.2	10.1	12.9
∑ PCDFs (pg/g wet weight)		13.6	2.42	7.65	14.4	10.3	17.7
∑ PCDD/Fs(pg/g wet weight)		35.7	20.4	25.1	31.6	20.3	30.6
∑ PCDD/Fs (pg TEQ/g wet weight)		**2.16**	**1.72**	**1.33**	**3.08**	**1.82**	**3.47**
18	PCB 81	241.0	137.5	47.5	58.8	4.2	104.2
19	PCB 77	1243.0	477.9	425.2	512.5	421.6	501.5
20	PCB 126	6.12	4.01	3.92	6.29	3.51	8.41
21	PCB 169	<0.828	1.07	1.72	3.04	<0.828	1.34
22	PCB-123	1404.0	486.3	307.9	41.9	47.5	4382.0
23	PCB-118	2478.5	77.2	45.2	62.0	41.5	5748.6
24	PCB-114	47.4	1086.3	1419.0	719.0	47.5	3041.5
25	PCB-105	176.7	41.5	44.2	104.2	4.15	3808.6
26	PCB-167	140.0	41.5	7.86	4.12	4.93	3676.2
27	PCB-156	144.3	108.6	41.9	32.2	7.15	1715.3
28	PCB-157	82.6	41.5	4.19	4.15	14.1	3815.3
29	PCB-189	143.3	41.5	108.9	41.5	9.2	1041.8
∑ NO PCBs (pg/g wet weight)		1490.5	620.5	478.3	580.6	429.9	615.4
∑ MO PCBs (pg/g wet weight)		4616.9	1924.5	1979.1	1009.0	176.1	27229.3
∑ dl PCBs(pg/g wet weight)		6107.4	2545.0	2457.4	1589.6	606.0	27844.7
∑ dl PCBs (pg TEQ/g wet weight)		**0.97**	**0.58**	**0.56**	**0.82**	**0.42**	**1.78**
∑ PCDD/F/dl PCBs (pg TEQ/g wet weight)		**3.13**	**2.3**	**1.89**	**3.9**	**2.24**	**5.25**
30	PCB-28	500.3	200.1	180.2	325.3	102.4	741.2
31	PCB-52	721.1	442.3	256.2	702.3	98.2	595.3
32	PCB-101	984.3	502.1	302.2	401.3	214.2	1172.3
33	PCB-153	1909.5	1279.4	988.7	3122.2	931.4	1429.7
34	PCB-138	1344.4	920.0	867.6	3062.2	731.4	1269.3
35	PCB-180	1120.3	941.2	1007.5	1809.2	707.1	1732.3
∑ NO PCBs (pg/g wet weight)		**6579.8**	**4285.1**	**3602.5**	**9422.4**	**2784.7**	**6940.2**

Table 3.6

Levels of PCDD/Fs and PCBs in the analyzed fish samples *(M.cephalus)*

SI No	**List of Compounds**	***M.cephalus***					
		SP1	**SP2**	**SP3**	**SP4**	**SP5**	**SP6**
1	2378-TCDD	0.07	<0.037	0.25	0.2	0.05	0.42
2	12378-PeCDD	0.83	<0.042	<0.042	0.23	0.62	1.1
3	123478-HxCDD	2.36	0.62	2.14	4.26	3.26	0.53
4	123678-HxCDD	1.25	4.44	0.13	1.24	2.36	2

5	123789-HxCDD	2.14	4.35	<0.044	<0.044	<0.044	0.9
6	1234678-HpCDD	0.2	0.22	0.96	23.57	13.56	4.32
7	OCDD	5.83	18.67	23.42	7.85	17.33	42.24
8	2378-TCDF	7.56	3.16	1.45	11.25	4.57	0.18
9	12378-PeCDF	4.85	27.24	5.45	4.37	7.86	14.67
10	23478-PeCDF	0.47	0.79	<0.102	0.41	0.54	0.7
11	123478-HxCDF	0.47	0.58	1.85	<0.034	1.26	8.24
12	123678-HxCDF	1.13	0.61	0.68	1.2	2.27	2.65
13	234678-HxCDF	3.16	0.04	0.50	1.36	1.47	1.95
14	123789-HxCDF	1.26	1.95	0.15	0.24	0.45	1.32
15	1234678-HpCDF	4.00	0.62	0.51	<0.043	12.36	<0.043
16	1234789-HpCDF	<0.031	0.07	0.39	23.65	<0.031	2.19
17	OCDF	<0.052	0.08	0.15	0.77	0.4	0.56
∑ PCDDs (pg/g wet weight)		12.7	28.4	26.9	37.4	37.2	51.5
∑ PCDFs (pg/g wet weight)		22.9	35.1	11.2	43.2	31.2	32.4
∑ PCDD/Fs(pg/g wet weight)		35.6	63.5	38.1	80.6	68.4	84.0
∑ PCDD/Fs (pg TEQ/g wet weight)		**3.16**	**2.72**	**1.21**	**3.12**	**2.9**	**4.03**
18	PCB 81	452	340.41	4.19	700.79	3.08	1738.2
19	PCB 77	2357.0	2625.7	15.7	2012.6	17.5	4261.7
20	PCB 126	5.32	4.57	0.66	5.68	0.5	7.71
21	PCB 169	<0.828	<0.828	<0.828	<0.828	<0.828	2.08
22	PCB-123	2438.1	1486.3	41.5	426.2	14.1	1586.3
23	PCB-118	2981.7	2908.9	41.9	805.1	33.8	4048.6
24	PCB-114	2714.1	2419.0	74.9	1374.9	37.5	4752.3
25	PCB-105	173.3	1071.5	44.9	1041.5	7.18	508.2
26	PCB-167	70.0	419.0	51.4	404.1	4.25	78.6
27	PCB-156	41.5	108.6	42.6	32.2	10.4	158.6
28	PCB-157	49.3	44.9	4.2	4.2	10.9	41.5
29	PCB-189	93.3	49.3	41.5	41.5	10.8	475.2
∑ NO PCBs (pg/g wet weight)		2815.0	2971.1	20.6	2719.7	21.2	6009.6
∑ MO PCBs (pg/g wet weight)		8561.4	8507.4	343.0	4129.7	128.9	11649.4
∑ dl PCBs(pg/g wet weight)		11376.4	11478.5	363.6	6849.4	150.1	17659.0
∑ dl PCBs (pg TEQ/g wet weight)		**1.18**	**1.1**	**0.1**	**1.13**	**0.08**	**2.13**
∑ PCDD/F/dl PCBs (pg TEQ/g wet weight)		**4.34**	**3.83**	**1.31**	**4.25**	**2.98**	**6.16**
30	PCB-28	725.4	323.4	482.4	525.4	80.2	1694.4
31	PCB-52	1042.4	584.5	509.5	1004.3	30.0	1478.5
32	PCB-101	1428.5	702.3	604.5	615.3	114.1	1865.5
33	PCB-153	2109.6	1422.6	1290.8	2820.4	908.4	1970.0
34	PCB-138	1646.5	1022.3	1279.8	3176.3	719.0	1667.6

35	PCB-180	1452.5	948.3	1196.7	1983.5	687.9	2233.7
	∑ NO PCBs (pg/g wet weight)	**8404.9**	**5003.3**	**5363.7**	**10125.1**	**2539.6**	**10909.6**

Table 3.7

Levels of PCDD/Fs and PCBs in the analyzed fish samples *(H.branchysoma)*

SI No	List of Compounds	*H.branchysoma*					
		SP1	**SP2**	**SP3**	**SP4**	**SP5**	**SP6**
1	2378-TCDD	0.31	0.21	<0.037	0.26	<0.037	0.12
2	12378-PeCDD	0.27	0.17	0.09	0.26	0.32	0.41
3	123478-HxCDD	0.17	0.17	0.21	1.94	3.64	3.83
4	123678-HxCDD	0.19	0.19	0.18	0.61	0.41	0.13
5	123789-HxCDD	0.12	0.12	0.17	0.48	0.23	0.33
6	1234678-HpCDD	7.41	7.41	9.99	6.2	0.24	<0.198
7	OCDD	15.54	15.54	<2.36	8.59	15	7.85
8	2378-TCDF	3.80	3.80	3.44	2.03	2.1	3.22
9	12378-PeCDF	10.24	0.24	0.53	10.37	1.53	0.72
10	23478-PeCDF	1.86	1.23	0.41	1.64	0.84	1.09
11	123478-HxCDF	1.33	0.13	0.30	3.00	1.02	2.03
12	123678-HxCDF	1.03	<0.036	0.33	<0.036	0.62	0.22
13	234678-HxCDF	0.07	0.07	0.11	1.34	0.23	0.43
14	123789-HxCDF	<0.032	<0.032	0.19	0.27	<0.032	0.11
15	1234678-HpCDF	0.11	0.11	0.64	0.65	0.33	5.12
16	1234789-HpCDF	0.15	0.15	0.09	0.32	3.13	0.24
17	OCDF	0.12	0.12	0.1	0.86	1.19	<0.052
∑ PCDDs (pg/g wet weight)		24.0	23.8	12.1	18.4	19.9	12.8
∑ PCDFs (pg/g wet weight)		18.7	5.87	6.15	20.48	11	13.17
∑ PCDD/Fs(pg/g wet weight)		42.7	29.68	18.23	38.82	30.86	25.97
∑ PCDD/Fs (pg TEQ/g wet weight)		**2.2**	**1.29**	**0.86**	**2.37**	**1.53**	**1.97**
18	PCB 81	67.9	24.5	33.8	33.8	23.8	100.4
19	PCB 77	302.7	123.6	256.9	402.6	14.2	400.2
20	PCB 126	0.71	0.49	0.34	0.8	0.65	0.6
21	PCB 169	<0.828	<0.828	<0.828	1.71	<0.828	<0.828
22	PCB-123	47.7	41.4	71.5	58.2	43.3	380.5
23	PCB-118	35.2	47.7	34.2	44.2	80.5	1104.2
24	PCB-114	1057.5	57.8	38.2	1375.3	170.8	370.8
25	PCB-105	374.6	34.0	34.2	48.6	43.4	475.2
26	PCB-167	44.1	40.4	10.8	70.5	6.0	140.5
27	PCB-156	220.4	54.1	7.5	41.5	41.5	48.4
28	PCB-157	41.1	14.4	10.7	4.90	250.9	103.8
29	PCB-189	74.9	41.6	38.2	294.2	10.8	36.2

∑ NO PCBs (pg/g wet weight)		371.3	148.6	291.1	438.9	38.8	501.9
∑ MO PCBs (pg/g wet weight)		1895.4	331.5	245.3	1937.4	647.2	2659.6
∑ dl PCBs(pg/g wet weight)		2266.7	480.1	536.4	2376.3	686.1	3161.5
∑ dl PCBs (pg TEQ/g wet weight)		**0.20**	**0.10**	**0.10**	**0.24**	**0.12**	**0.23**
∑ PCDD/F/dl PCBs (pg TEQ/g wet weight)		**2.4**	**1.4**	**0.97**	**2.61**	**1.65**	**2.21**
30	PCB-28	651.3	287.2	123.2	792.2	147.2	1277.7
31	PCB-52	869.3	302.1	132.1	688.2	168.3	1628.0
32	PCB-101	609.3	442.1	325.2	597.3	212.2	1257.5
33	PCB-153	838.3	330.1	212.3	842.1	108.4	2412.2
34	PCB-138	515.6	763.2	398.1	1932.1	396.1	1430.2
35	PCB-180	559.6	642.3	412.2	1670.2	400.1	1942.2
∑ NDL PCBs (pg/g wet weight)		**4043.5**	**2767.1**	**1603.2**	**6522.2**	**1432.5**	**9947.9**

Table 3.8

Levels of PCDD/Fs and PCBs in the analyzed fish samples *(O.mossabicus)*

SI No	**List of Compounds**	**O.mossabicus**					
		SP1	**SP2**	**SP3**	**SP4**	**SP5**	**SP6**
1	2378-TCDD	0.40	0.32	0.07	0.14	0.12	0.23
2	12378-PeCDD	<0.042	<0.042	0.09	0.18	0.30	0.21
3	123478-HxCDD	<0.04	0.18	0.91	<0.04	<0.04	<0.04
4	123678-HxCDD	<0.033	<0.033	0.48	0.2	<0.033	<0.033
5	123789-HxCDD	<0.044	<0.044	0.20	0.15	<0.044	<0.044
6	1234678-HpCDD	0.60	0.60	1.50	15.13	3.25	0.33
7	OCDD	15.17	15.42	14.42	24.22	18.97	41.53
8	2378-TCDF	0.61	0.64	0.11	1.56	1.14	2.75
9	12378-PeCDF	0.45	0.47	0.42	1.52	3.44	2.30
10	23478-PeCDF	0.62	0.51	0.22	0.51	<0.102	<0.102
11	123478-HxCDF	0.54	<0.034	0.07	0.09	<0.034	<0.034
12	123678-HxCDF	<0.036	<0.036	0.35	<0.036	<0.036	0.05
13	234678-HxCDF	<0.028	<0.028	0.27	0.15	0.12	<0.028
14	123789-HxCDF	<0.032	<0.032	0.20	<0.032	<0.032	<0.032
15	1234678-HpCDF	<0.043	0.40	0.42	0.31	1.45	2.15
16	1234789-HpCDF	<0.031	<0.031	0.20	0.22	0.14	0.24
17	OCDF	0.09	0.12	0.15	0.54	1..00	0.70
∑ PCDDs (pg/g wet weight)		16.17	16.52	17.66	40.02	22.67	42.35
∑ PCDFs (pg/g wet weight)		2.3	2.15	2.42	4.91	7.36	8.24
∑ PCDD/Fs(pg/g wet weight)		18.47	18.67	20.08	44.93	30.02	50.58
∑ PCDD/Fs (pg TEQ/g we weight)		**0.79**	**0.65**	**0.52**	**0.91**	**0.76**	**0.88**

18	PCB 81	104.2	27.1	71.5	100.4	4.2	40.3
19	PCB 77	361.4	230.1	312.5	400.2	15.7	302.5
20	PCB 126	0.61	0.40	0.62	0.60	0.40	0.85
21	PCB 169	<0.828	<0.828	<0.828	<0.828	<0.828	1.71
22	PCB-123	41.1	247.4	82.9	124.6	166.1	317.3
23	PCB-118	33.8	38.0	95.5	125.7	167.6	476.4
24	PCB-114	748.4	775.2	115.5	224.6	299.5	950.5
25	PCB-105	441.5	38.1	68.1	134.8	179.7	101.6
26	PCB-167	37.5	70.8	80.9	154.3	205.7	15.7
27	PCB-156	254.0	40.5	108.2	127.9	170.5	31.7
28	PCB-157	47.4	46.5	28.8	12.6	16.8	8.31
29	PCB-189	77.4	108.9	83.2	124.6	166.1	95.0
∑ NO PCBs (pg/g wet weight)		466.3	258.0	384.8	501.9	20.3	345.4
∑ MO PCBs (pg/g wet weight)		1681.0	1365.4	663.0	1029.0	1372.0	1996.5
∑ dl PCBs(pg/g wet weight)		2147.3	1623.4	1047.7	1530.9	1392.3	2341.9
∑ dl PCBs (pg TEQ/g wet weight)		**0.20**	**0.14**	**0.16**	**0.19**	**0.11**	**0.24**
∑ PCDD/F/dl PCBs (pg TEQ/g wet weight)		**0.99**	**0.78**	**0.68**	**1.1**	**0.87**	**1.12**
30	PCB-28	414.0	136.3	163.2	251.2	124.3	142.6
31	PCB-52	573.9	148.7	152.4	282.2	142.6	132.7
32	PCB-101	564.0	206.2	202.1	484.8	182.2	272.7
33	PCB-153	344.0	226.2	150.3	892.1	202.2	592.7
34	PCB-138	134.9	406.3	392.1	792.1	259.9	540.7
35	PCB-180	374.0	381.3	385.2	855.2	325.4	604.6
∑ NO PCBs (pg/g wet weight)		**2404.7**	**1505.2**	**1445.3**	**3557.7**	**1236.6**	**2286.1**

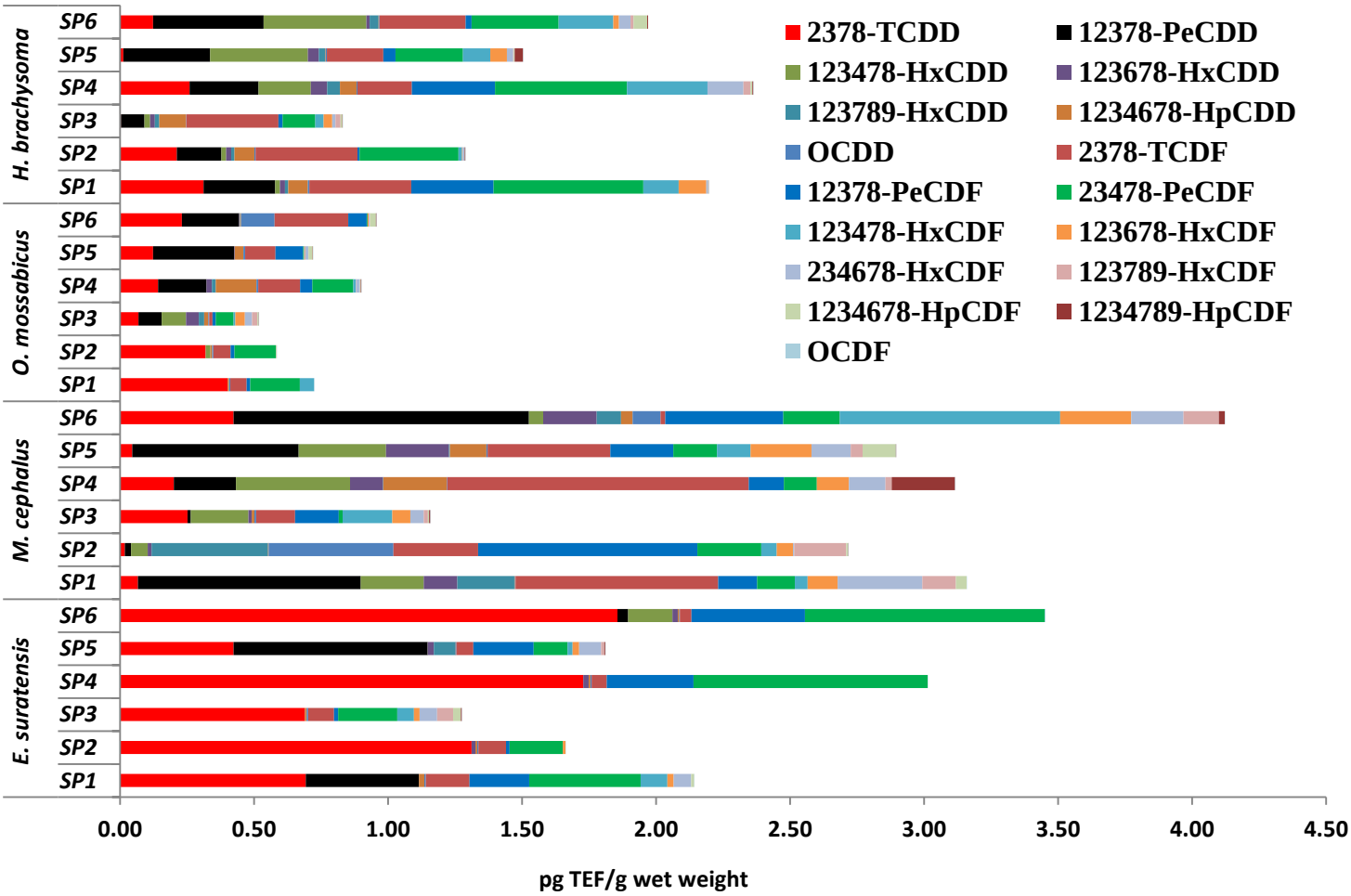


Fig. 3.4. Levels of PCDD/Fs in the analyzed fish samples

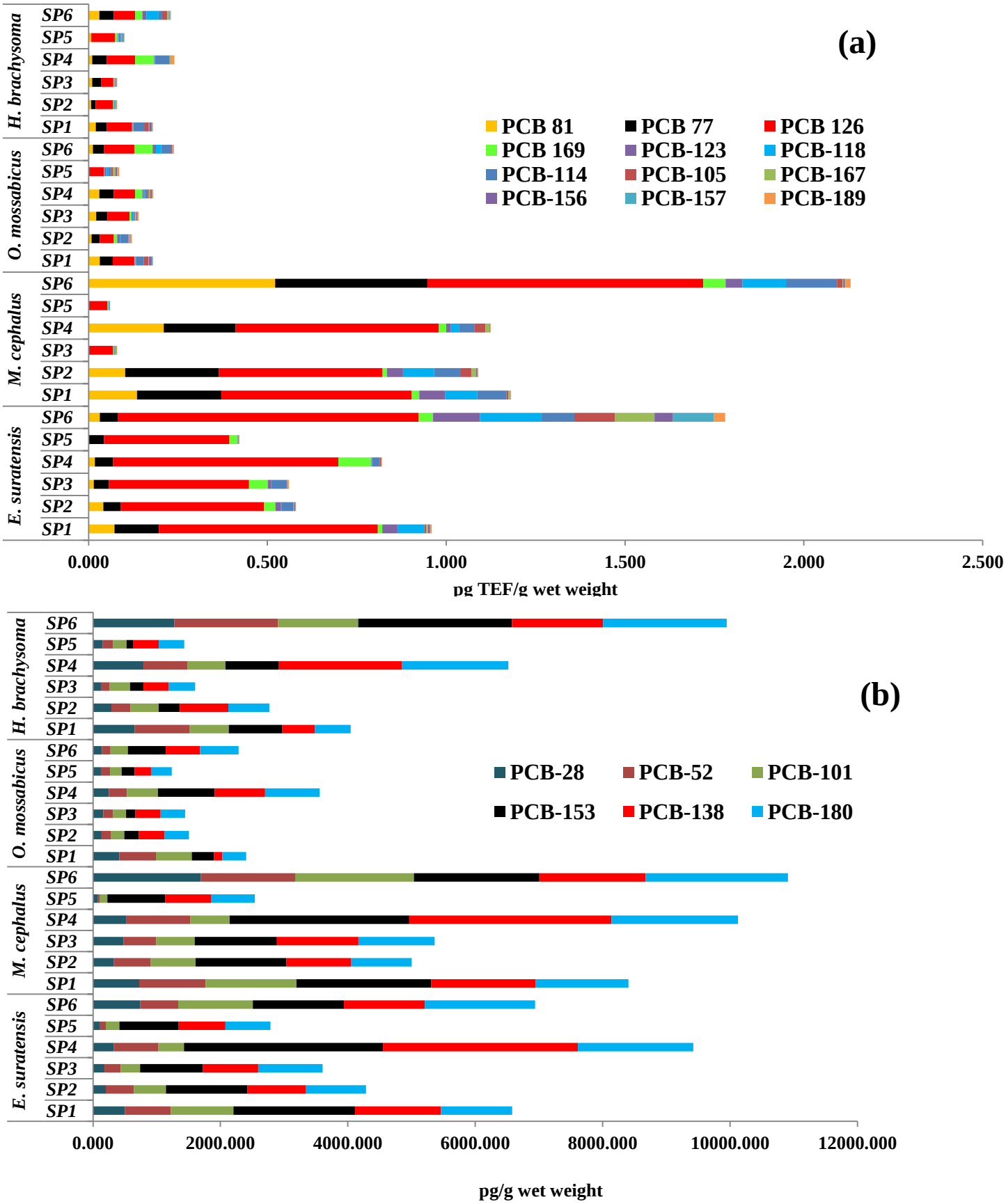


Fig. 3.5. Levels of PCBs observed in analyzed fish species (a) dl-PCBs (b) ndl-PCBs samples

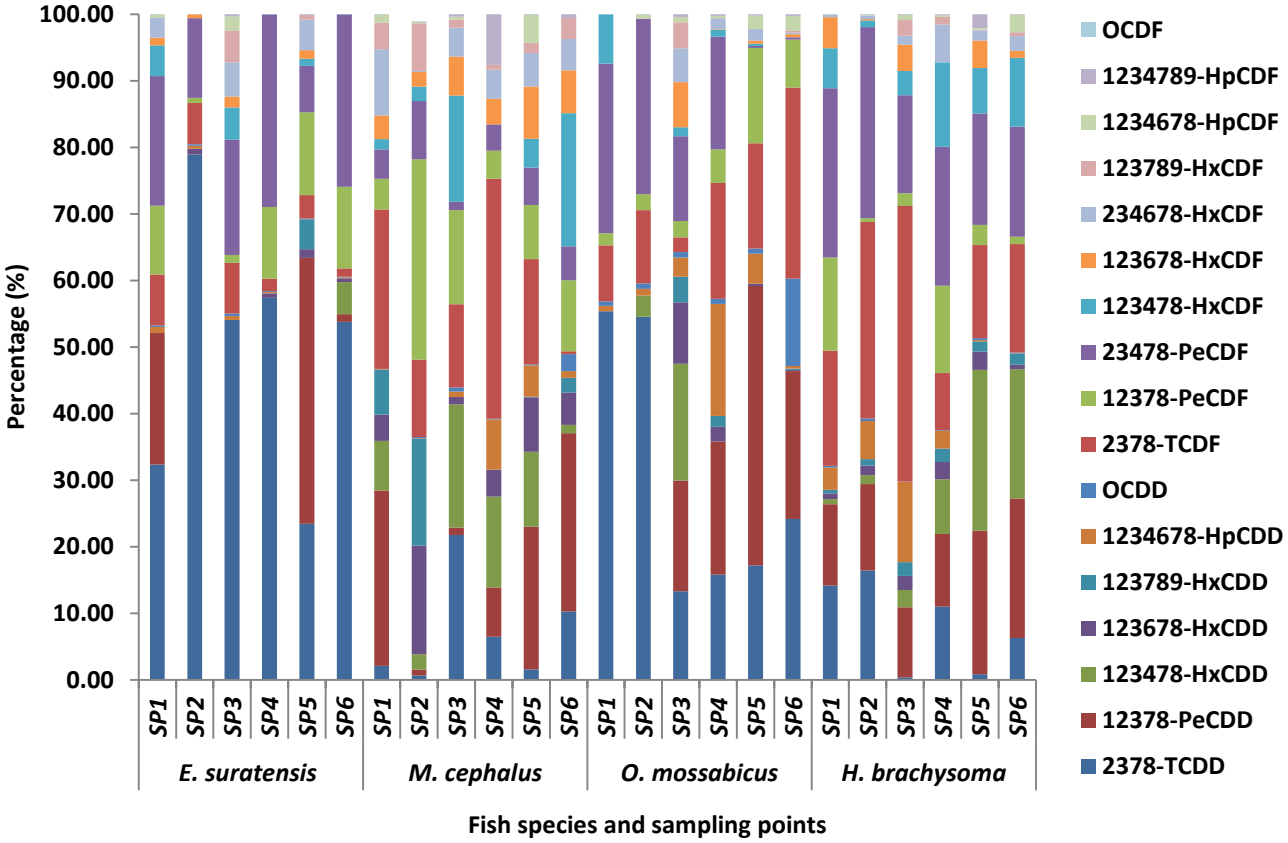


Fig. S9. Percentage contribution of individual congeners to ∑**PCDD/Fs (pg TEQ/g wet weight)**

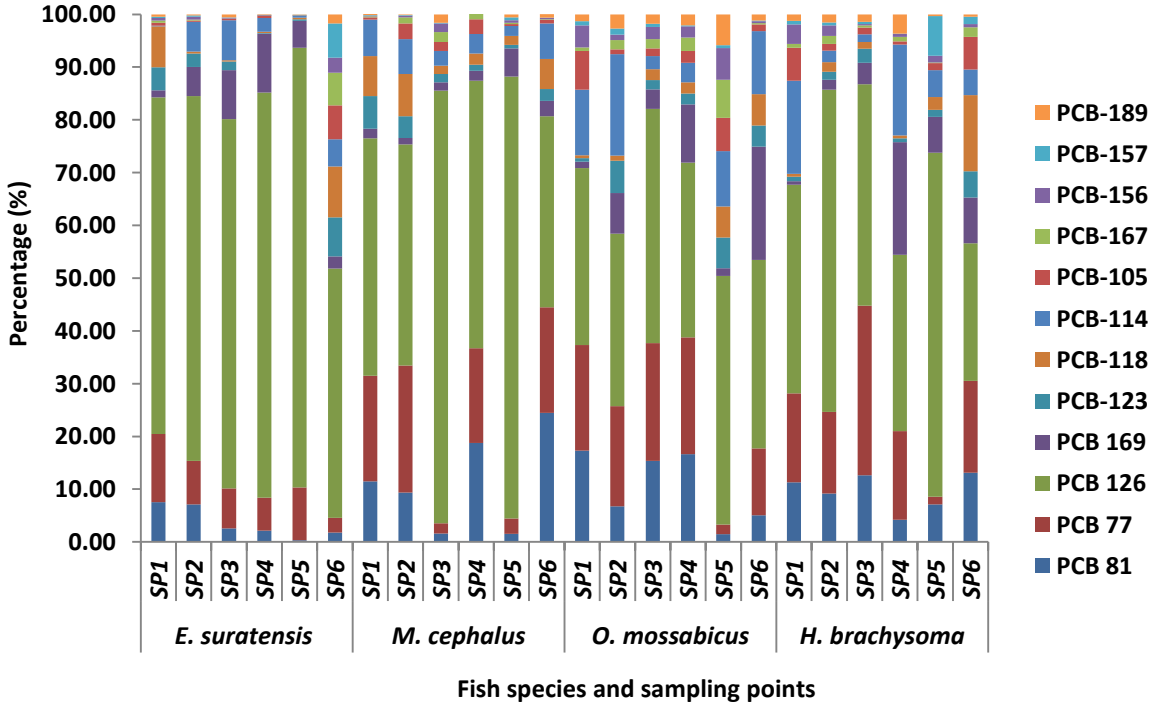


Fig. 3.6. Percentage contribution of individual congeners to ∑**dl-PCBs (pg TEQ/g wet**

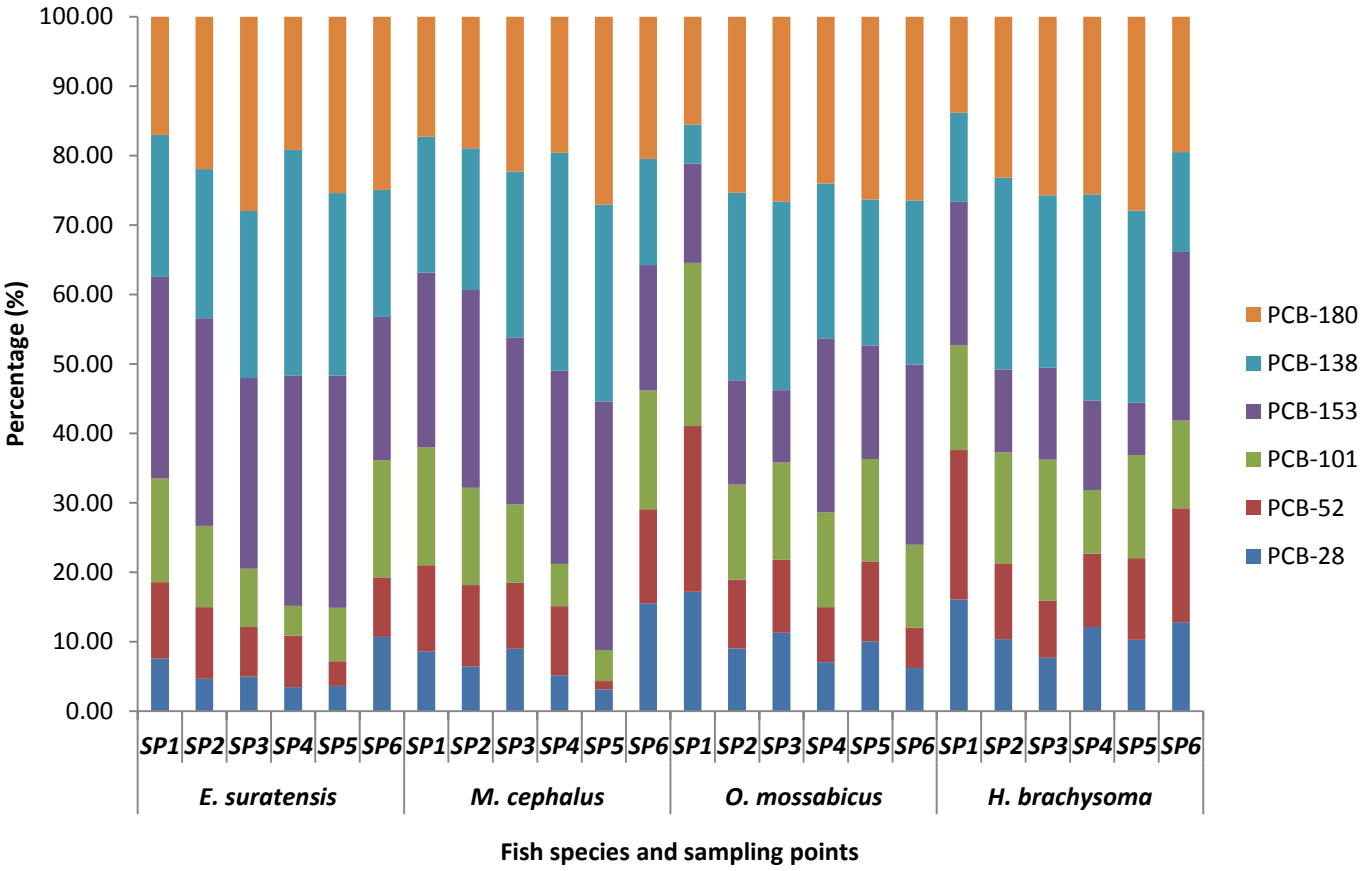


Fig. 3.7. Percentage contribution of individual congeners to ∑ndl-PCBs (pg/g wet weight)

3.4.4. Trends of PCDD/Fs and PCBs in fish control samples

The levels of PCDD/Fs and PCBs in samples of E. suratensis collected from a river in a non-industrial area (field control) and the open market (market control) were analyzed (**Table 3.9**). The group-wise comparison of the cumulative congener concentration is shown in **Fig. 3.8**. For PCDD/Fs, the average TEQ of control samples was 0.42 (field control) and 0.51 (market control) pgTEQ/g w.w, which is eight times lower than the most polluted sampling point (3.47) in the hotspot region. Notably, in both control samples, the most toxic congeners such as 2378 TCDD and 12378 PeCDD (having TEF=1) were either not detected or were below the limit of quantification, while these two congeners registered a major contribution to the sum TEQ in POPs hotspot samples. Regarding dl-PCBs, the levels varied between 0.04 to 0.063 with an average of 0.06 pg TEQ/g w.w (field control) and 0.007 to 0.05 with an average of 0.04 pg

TEQ/g w.w (market control). A 30-fold decrease in the dl-PCBs sum TEQ in the average control samples compared to the most polluted sampling point (1.78) can be concluded from the studies. Similarly, a larger deviation (about 85-fold increase) in ndl-PCBs levels in POPs hotspot samples (9.42 ng/g) compared to control samples (0.11 ng/g) can also be observed.

Table 3.9
Levels of PCDD/Fs and PCBs in control samples of E.suratensis

SI No	List of Compounds	TEF	Field Control	Market control
1	2378-TCDD	1.000	<0.037	0.20
2	12378-PeCDD	1.000	<0.042	<0.042
3	123478-HxCDD	0.100	0.61	0.80
4	123678-HxCDD	0.100	0.43	0.56
5	123789-HxCDD	0.100	<0.044	<0.044
6	1234678-HpCDD	0.010	0.44	0.42
7	OCDD	0.000	<2.36	<2.36
8	2378-TCDF	0.100	0.17	0.12
9	12378-PeCDF	0.030	0.09	0.04
10	23478-PeCDF	0.300	0.42	0.14
11	123478-HxCDF	0.100	0.17	0.20
12	123678-HxCDF	0.100	0.16	0.15
13	234678-HxCDF	0.100	0.15	0.03
14	123789-HxCDF	0.100	0.27	0.30
15	1234678-HpCDF	0.010	0.50	0.39
16	1234789-HpCDF	0.010	0.11	0.12
17	OCDF	0.000	0.36	0.36
∑ PCDDs (pg/g dry weight)			2.35	3.6
∑ PCDFs (pg/g dry weight)			2.42	1.85
∑ PCDD/Fs(pg/g dry weight)			4.76	5.45
∑ PCDD/Fs (pg TEQ/g dry weight)			0.42	0.51
18	PCB 81	0.00030	1.13	0.41
19	PCB 77	0.00010	0.78	1.73
20	PCB 126	0.10000	0.30	<0.191
21	PCB 169	0.03000	<0.828	<0.828
22	PCB-123	0.00003	1.76	3.75
23	PCB-118	0.00003	2.40	3.63
24	PCB-114	0.00003	0.31	1.58
25	PCB-105	0.00003	1.30	0.28
26	PCB-167	0.00003	0.48	0.94
27	PCB-156	0.00003	0.39	0.46
28	PCB-157	0.00003	0.52	1.05

29	PCB-189	0.00003	<0.167	0.22
∑ NO PCBs (pg/g dry weight)			2.5	2.34
∑ MO PCBs (pg/g dry weight)			7.32	11.9
∑ dl PCBs(pg/g dry weight)			9.82	14.2
∑ dl PCBs (pg TEQ/g dry weight)			0.06	0.04
∑ PCDD/F/dl PCBs (pg TEQ/g dry weight)			0.48	0.56
30	PCB-28	NA	0.03	0.01
31	PCB-52		0.02	0.03
32	PCB-101		0.02	0
33	PCB-153		0	0.08
34	PCB-138		0	0.01
35	PCB-180		0	0.01
∑ NO PCBs (pg/g dry weight)			0.07	0.14

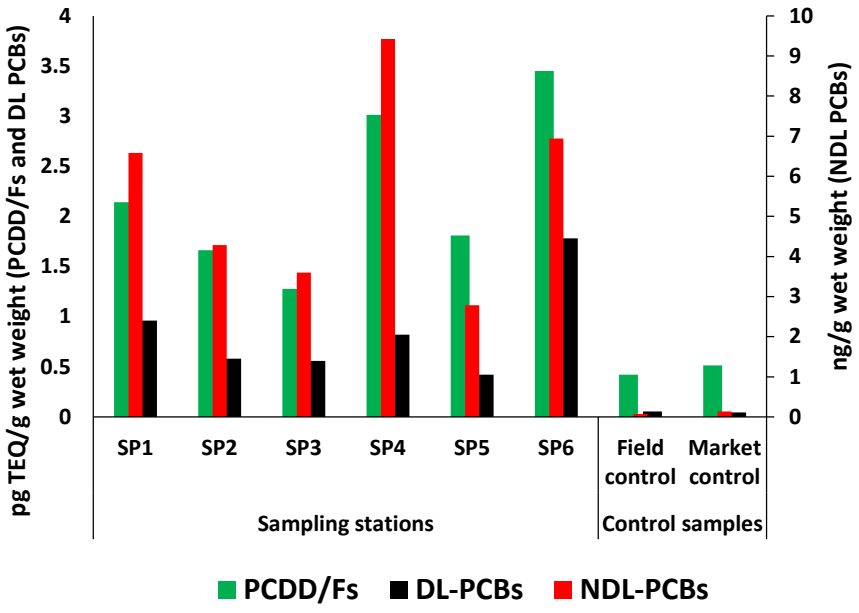


Fig.3.8. Comparison plot of different congener groups: industrial hotspot sample vs control site

3.4.5. Comparative evaluation of present status of hotspot vis-à-vis global scenario

A thorough examination of prior research and reports concerning the POPs hotspot revealed instances and evidence of harmful effects of pollution in this area, including frequent fish deaths

and deterioration in air and water quality quality (Anjusha et al., 2020; Charuvilayil et al., 2011; Suchitra, 2020). The majority of research conducted in the area focused on contamination from pesticide-based POPs such as DDT, Dicofol, etc., with only a few studies reporting on dl-POPs (Deepa et al., 2008; Stringer et al., 2003; Sujatha et al., 1994. As part of the global dioxin surveillance project, IPEN reported the sole available study in the hotspot region, which revealed four times higher levels of dioxins (13.91 pg TEQ/g fat) and PCDD/F/dl-PCBs (15.08 pg TEQ/g fat) in eggs of free-range chickens than EU regulations permit (Petrlik et al., 2005. This study also suggested that the congener profiles observed in the egg samples were akin to those obtained from samples taken from an outdated pesticide stockpile in Vikuge, Tanzania, confirming the possibility that pesticide production facilities could serve as sources of dioxins**.** **Fig.3.9.** depicts the peak total TEQ/concentration of congener groups (i) PCDD/Fs, (ii) dl-PCBs, (iii) ndl-PCBs observed in sediment (a) and fish (b) samples from different sampling points. The levels of contaminants (Σ PCDD/Fs + dl-PCBs) in sediment samples from three sampling points (SP1, SP6, SP4) exceeded the action level of 21.5 pgTEQ/g recommended by Canada for eco-toxicological assessment. In contrast, the levels were well below the remedial action level set by Japan (150 pgTEQ/g) and the Netherlands (1000 pgTEQ/g) based on human risks (Suzuki et al., 2016). Fish and sediment samples taken from SP6 exhibited higher concentrations of all three categories of dl- POPs, indicating a strong confluence of emission source - environmental - food nexus towards bioaccumulation. The cumulative PCDD/Fs and dl-PCBs levels varied between 0.68 and 6.16 pgTEQ/g wet weight in the analyzed fish samples from different sampling points, remaining below the EU maximum of 6.5 pg TEQ/g wet weight. Although Σ dl-POPs were not alarming, the levels of dioxins obtained were higher/equal in M. cephalus and E. suratensis than the EU maximum level of 3.5 pg TEQ/g for fishery products. The limited surveillance of dl-POPs in this region may be due to inadequate facilities and cost-effective validated analytical methods. From this perspective, the developed analytical workflow can serve as an excellent tool for enabling low-cost routine monitoring of POPs hotspots worldwide. **Fig. 3.9 (c)** illustrates a comparison plot of sediment levels in the current hotspot under investigation with other hotspots around the world. Although IPEN identified forty hotspots of Persistent Organic Pollutants (POPs) globally, surveillance studies were constrained. The selection of these POPs hotspots was based on the presence of various potential emission sources of POPs in these areas and a limited number of sample analyses. Among the available studies, the majority focused on the most toxic

congener group dioxin and furans while PCBs are less studied. The highest reported levels of POPs were found in the Pacer Ivy area of Vietnam (1.50×10^3 pgTEQ/g) due to historical Agent Orange usage. In comparison to the limited global data available, the extent of contamination at the present hotspot is moderate. The scarcity of surveillance data underscores the urgency of developing viable analytical methods for regular monitoring of these hotspots to ensure human health security.

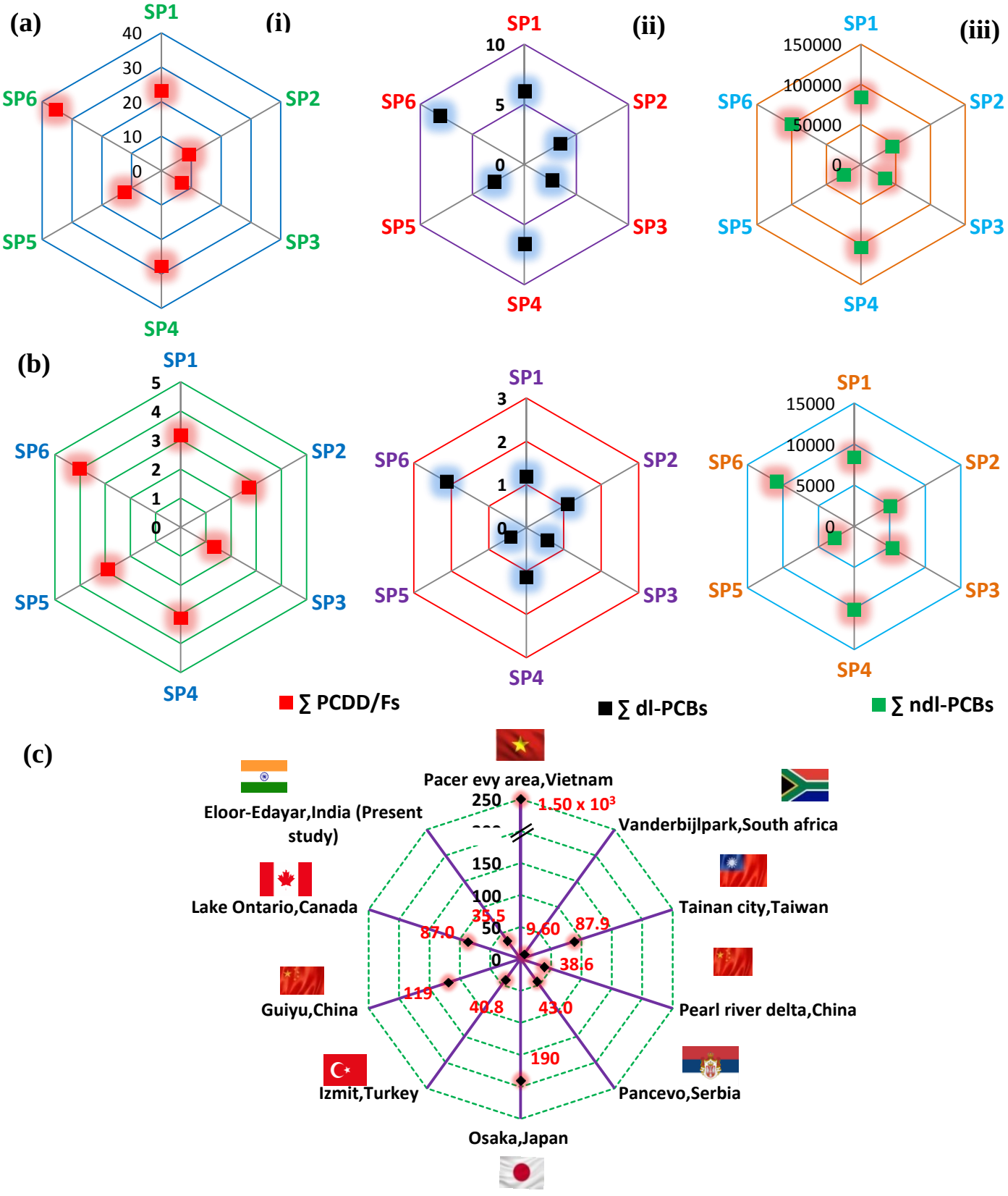


Fig. 3.9. Present status of hotspot in terms of (i) dioxins (ii) dl-PCBs (iii) ndl-PCB: (2a) Sediment samples (d.w basis) (1b) fish samples (w.w basis) (1c) PCDD/Fs levels observed in sediment samples of various hotspots around the world (pg TEQ/g dw basis) (Bhavsar et al., 2010; Chang et al., 2010; Kaisarevic et al., 2011; Karademir et al., 2013; Kishida et al., 2010; Liu et al., 2022, 2023; Nieuwoudt et al., 2009; Van Thuong et al., 2015).

3.4.6. Correlation analysis and bio accumulation trends of contaminants

The benthopelagic nature of the fish species analyzed in this study could result in the long-term accumulation of sediment-bound contaminants in their tissues. To explore possible correlations, regression analysis was conducted by estimating Pearson's correlation coefficient between the levels of PCDD/Fs, dl-PCBs (WHO2005 TEQs), and Σndl-PCBs in fish species and the corresponding sediment levels at each sampling point. The regression analysis revealed a strong overall positive correlation between the contaminant levels in the analyzed fish and sediment samples, irrespective of the contaminant group/subgroup. This positive correlation, supported by various measurable indices such as correlation coefficients and statistical significance ($p < 0.05$) (**Fig.3.10**), suggests high bioaccumulation of these contaminants from sediment into fish tissues. This observation is consistent with a similar correlation observed between mussel fish and sediment samples from Kentucky Lake, USA (Loganathan et al., 2008). Approximately 83.3% of the regression analysis data were statistically significant with a p-value < 0.05. These findings can serve as a preliminary tool to assess the tendency for bioaccumulation of contaminants from sediment into fish. Furthermore, to elucidate congener/species-related bioaccumulation trends, a more in-depth study was conducted by estimating Biota Sediment Accumulation Factor (BSAF) using absolute concentrations of contaminant groups instead of levels expressed in terms of TEQ. The TOC levels varied between 0.011 and 0.019 g/g dw for sediment samples. The estimated BSAF values with absolute concentrations of individual congeners showed that higher order bio magnification of dl-PCBs occurred in all fish species compared to PCDD/Fs and ndl-PCBs (**Fig.3.11**). This analysis demonstrated the crucial role of BSAF studies in identifying the underlying mechanistic pathways involved in congener-wise accumulation trends and their overall impact on toxicological aspects.

The degree of chlorination and the position in the aromatic ring, along with other physicochemical properties, determine the bioaccumulation tendencies of dl-POPs congeners (Stohs, 2014).. PCB congeners with high chlorine substitution as well as lower ortho substitution have poor volatility and water solubility, resulting in higher affinity for sediments (IARC, 2015). For PCDD/Fs, BSAF values ranged from 0.019 to 0.092. Higher BSAF values for PCDD/Fs were observed in SP2 for all analyzed fish species compared to other sampling points. Samples of M. cephalus (0.0920) and H. barchysoma (0.0810) from SP2 showed higher PCDD/Fs-BSAF

values among the fish samples analyzed. Higher BSAF values at SP2 can be attributed to the highest TOC level observed in the sediment samples from SP2 (0.019 g/g dw of sediment), compared to other sampling points. The low BSAF values observed in SP4 (0), SP5 (0.033), and SP6 (0.038) reiterated the critical role of bioavailability, hydrophobicity, and feeding pattern in understanding the accumulation pattern. For dl-PCBs, the calculated BSAF values ranged from 4.00×10^{-3} to 0.671. Additionally, higher BSAF for ndl-PCBs was observed in E. suratensis at all sampling points, ranging from 0.0180 to 0.0710. The BSAF values obtained indicated a higher accumulation of PCDD/Fs in O. mossabicus and M. cephalus, and dl and ndl-PCBs in E. suratensis species, suggesting the need for detailed studies to understand the factors contributing to the trends in bioaccumulation, such as contaminant metabolic rates and feeding nature.

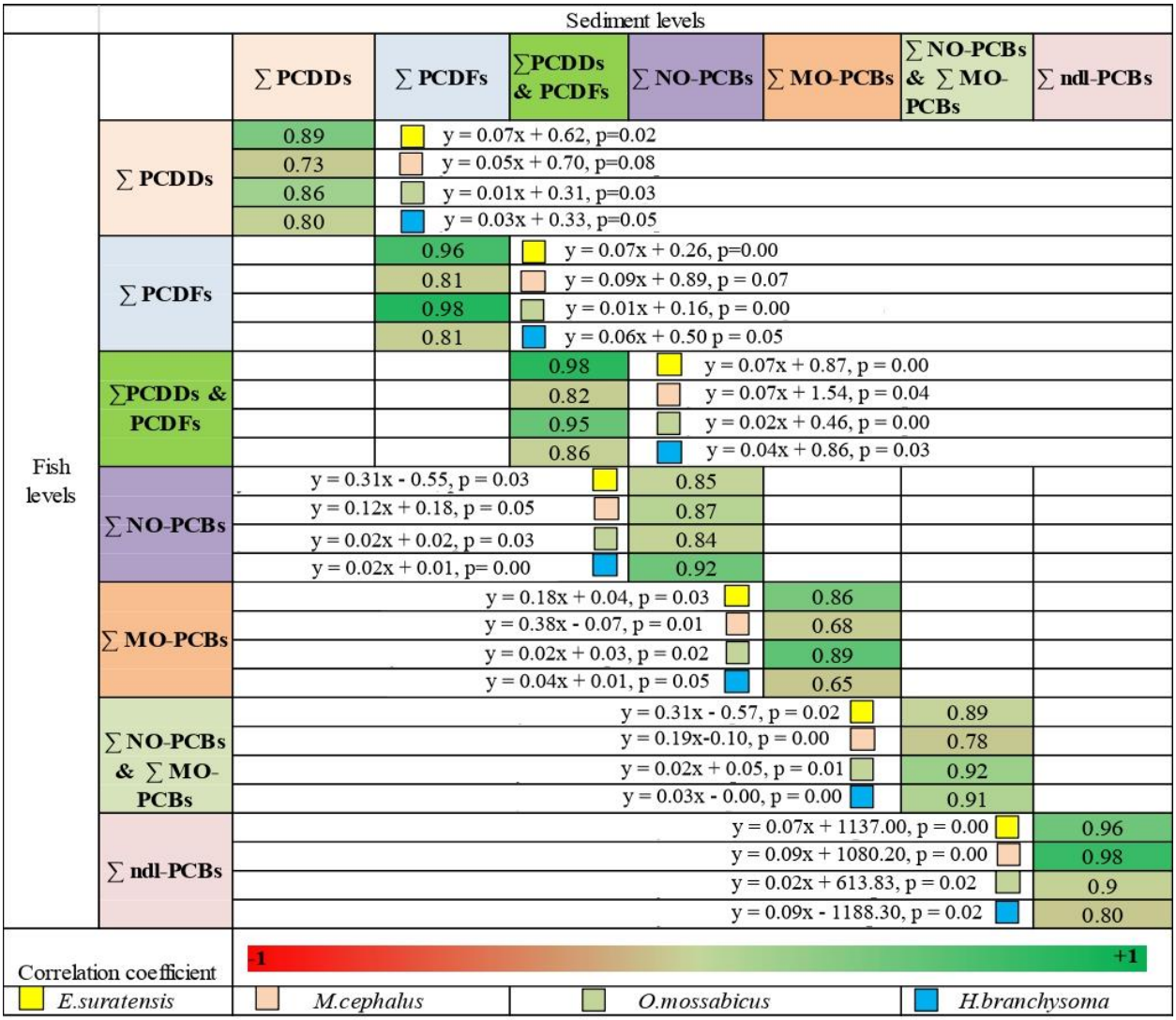


Fig. 3.10 Regression analysis trends between fish and sediment samples

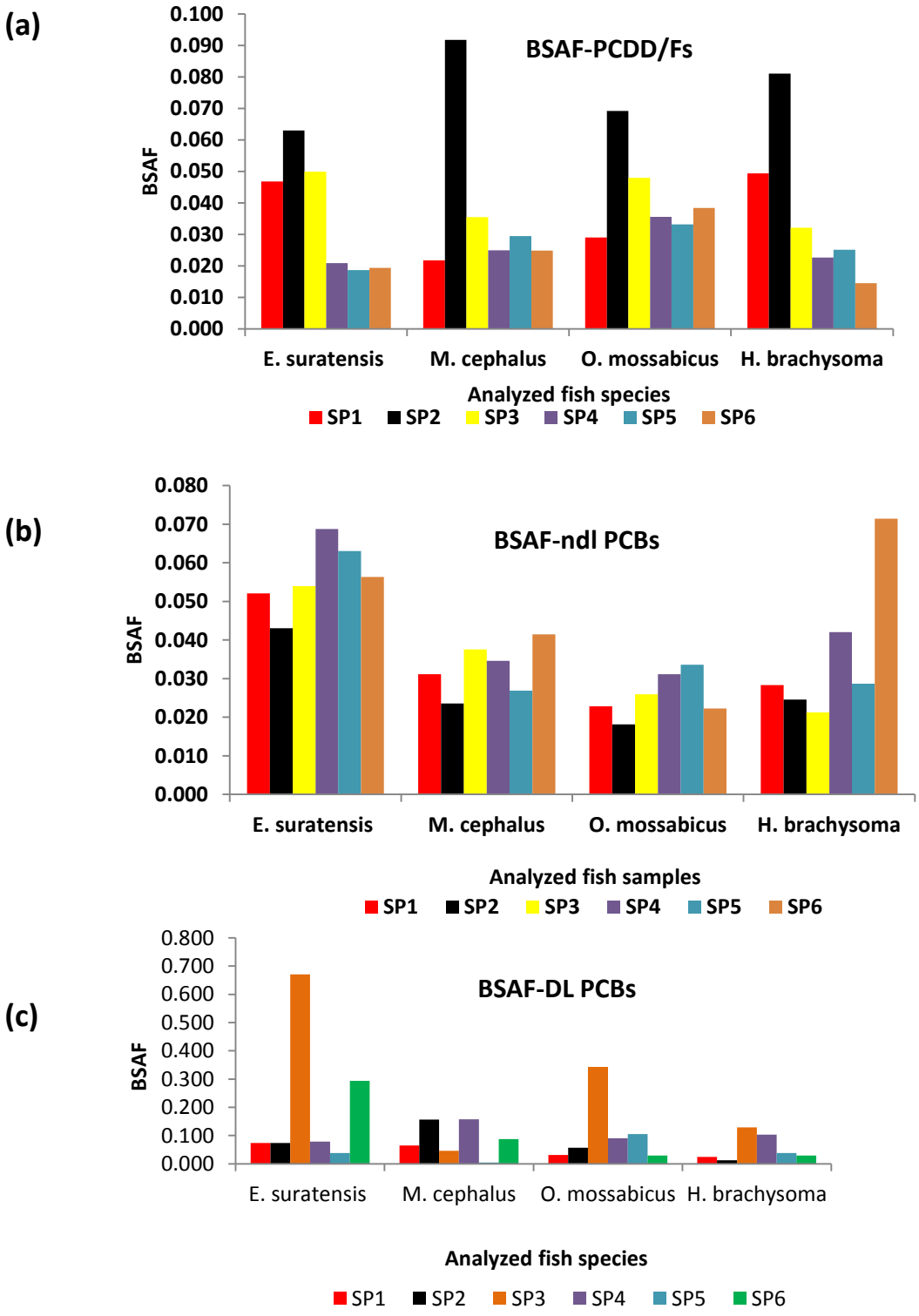


Fig. 3.11 Biotata sediment accumulation factors for different fish species (a) BSAF PCDD/Fs, (b) BSAF dl - PCBs and (c) BSAF ndl- PCBs

3.4.7. Health risk assessment of fish consumers in the study area

The health risk assessment for fish consumers in the study region was conducted by calculating the combined dietary intakes of PCDD/Fs and dl-PCBs. The World Health Organization (WHO) recommended a tolerable daily intake (TDI) of 1–4 pgTEQ per kg body weight per day, while the European Food Safety Authority (EFSA) suggested a more cautious tolerable weekly intake (TWI) level of 2 pgTEQ per kg body weight per week (Leeuwen et al., 2000; EFSA CONTAM Panel, 2018). Daily fish consumption in rural South India is estimated to be 80.7 g, with an average adult bodyweight of 60 kg taken into account for the estimation of species-specific daily intake of fish consumers (NIN, 2020).. The calculated daily intake varied between 0.89 and 8.41 pgTEQ per kg body weight per day across different fish species and sampling locations. A comparative analysis (**Fig. 3.12**) of the results of the present study in relation to the WHO-recommended higher TDI value (4 pg TEQ per kg body weight per day) suggested the likelihood of an increased risk for consumers, particularly in the case of M. cephalus and E. suratensis. Consumption of M. cephalus could result in daily intake levels ranging from 1.67 to 8.41 pgTEQ per kg body weight per day, with four out of six sampling sites reporting the possibility of a higher daily intake compared to the TDI. Similarly, sampling points SP1, SP4, and SP6 reported higher daily intake values for E. suratensis, ranging from 4.12 to 7.03. Despite differences in fish species and sampling sites, the calculated weekly intake values for E. suratensis (17.5–49.2), M. cephalus (11.7–58.9), H. brachysoma (8.54–24.9), and O. mossabicus (6.23–11.3) were observed to exceed the more conservative EFSA TWI limit of 2 pgTEQ per kg body weight per week. Comparable trends were reported in Baltic fish species elsewhere, with weekly intake values 8–24 times higher compared to the EFSA TWI, consistent with the findings of the present study (Mikolajczyk et al., 2021). Conversely, the daily intake associated with Mediterranean swordfish was relatively low compared to the present study, ranging from 0.910 to 3.15 (Mehouel, 2021). Overall, these findings indicate a potential health risk for consumers of fish in the POPs hotspot region of global importance and underscore the necessity for regular monitoring with affordable confirmatory tools, establishment of consumption guidelines, and scientific pollution control and remediation measures.

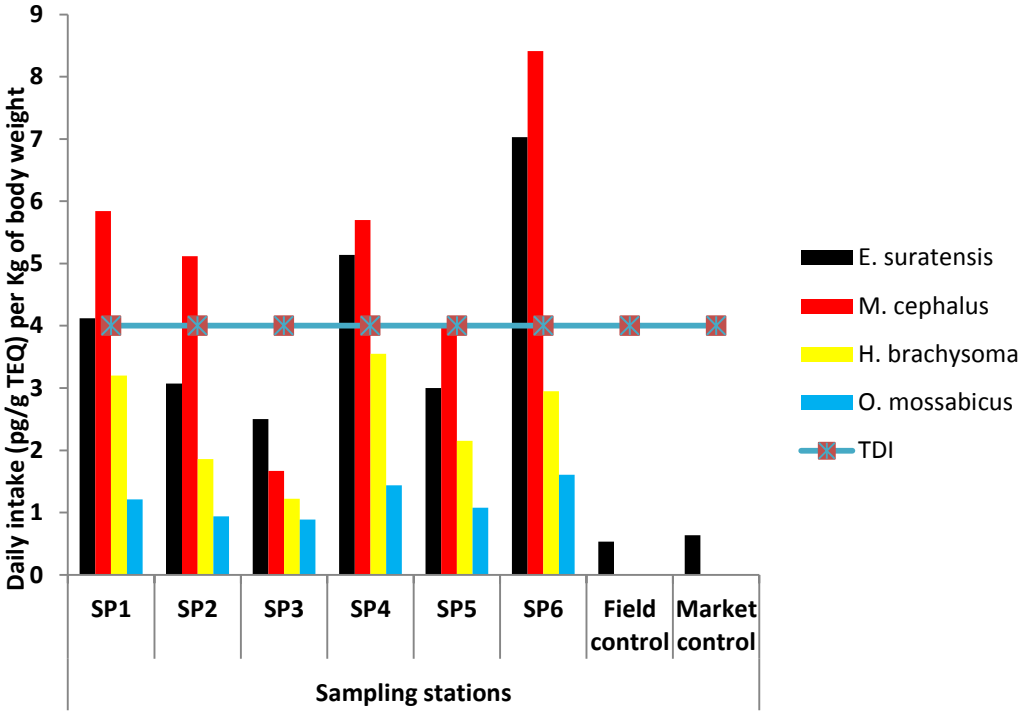


Fig. 3.12 Health risk assessment to fish consumers in the study area

3.5. Conclusions

In summary, this study introduces a cost-effective integrated workflow designed to evaluate the environmental and health risks linked with dioxin-like Persistent Organic Pollutants (dl-POPs) in industrial hotspot regions. Through the examination of fish and sediment samples from a significant dl-POPs hotspot, the Eloor-Edayar industrial belt in India, valuable insights into the prevalence and sources of dl-POPs in the ecosystem are provided. The study highlights that dl-POPs primarily form through precursor pathways, indicating the potential release of chlorinated precursor species from the surrounding industrial area. Fish samples from the hotspots exhibit notably higher levels of polychlorinated dibenzo-p-dioxin/furans (PCDD/Fs) and polychlorinated biphenyls (PCBs) compared to control sites, emphasizing the importance of regular surveillance in hotspot regions. Correlation analysis supports the transfer of contaminants into the food chain, further underlining the necessity for ongoing monitoring efforts. Moreover, the estimated weekly intake of dl-POPs from fish consumption in the study region surpasses the maximum levels established by the European food safety authority, implying a potential health risk for fish

consumers. Thus, it is imperative to implement periodic surveillance using user-friendly and validated confirmatory tools to safeguard both human health and the environment. The identified congener fingerprints, correlation patterns, and source attribution of dl-POPs serve as valuable metadata for understanding the fate of these compounds in the environment and devising effective mitigation strategies. Looking ahead, this study sets the stage for future advancements in dl-POPs research. Expanding the application of the GC-MS/MS-based workflow to other matrices and emission sites would enable a comprehensive assessment of the environmental-food-human health nexus and support stakeholders in making informed decisions and formulating global strategies to mitigate the risks associated with dl-POPs. Ultimately, the integration of cost-effective analytical methodologies and field-deployable workflows will contribute to the efficient monitoring and management of dl-POPs, ensuring a safer and healthier environment for present and future generations.

Chapter

4

Exploring the potential of coconut shell-based activated carbon as an efficient sorbent for dioxins and PCBs decontamination from fish oil

4.1. Abstract

This chapter presents an innovative and cost-effective approach utilizing coconut shell-based chemically activated carbon (AC) as a highly efficient sorbent for decontaminating dioxins and PCBs from fish oil. The synthesized AC employing KOH as activation agent, underwent thorough characterization through SEM imaging and N_2 adsorption-desorption physisorption analysis. The results revealed a well-developed pore structure on the surface and a significant increase in surface area (390.01 m^2/g), along with improved textural properties post-activation. The study delves into the often-overlooked kinetic and adsorption characteristics of individual dioxins and non-ortho PCBs, which are crucial for understanding the efficiency of AC. The congeners generally exhibited Langmuir > Freundlich > Temkin adsorption trends, while kinetic models indicated pseudo-second order > pseudo-first order > intra-particle diffusion. In the latter part of the study, the effectiveness of the synthesized AC in decontaminating these contaminants from fish oil matrices was explored. Optimized Fish oil: AC ratio (1: 0.1) achieved an impressive 85-97% decontamination rate for all congeners. Furthermore, the method's efficacy was confirmed by evaluating fish oil quality parameters such as fatty acid profile, FFA value, and acid values before and after the decontamination process. The retention of quality parameters post-decontamination highlights the method's effectiveness in purifying fish oil from these contaminants, without compromising its quality.

4.2. Introduction

Poly chlorinated dibenzo dioxins/furans (PCDD/Fs) and polychlorinated biphenyls (PCBs), recognized as three key members of the infamous "dirty dozen" of persistent organic pollutants, have become a focal point of concern due to their detrimental impact on human health and the environment (Stockholm Convention, 2004). The potential health risks associated with ultra-trace level of these contaminants demands regular surveillance in different matrices with superior confirmatory analytical methodologies (Bertazzi et al., 2001; van Gerwen et al., 2023; VoPham et al., 2020). International agencies, such as the Environmental Protection Agency and the European Union, have established strict regulations and analytical protocols to monitor this class of pollutants in food and environmental matrices (USEPA, 2013; European Union, 2017).

Overtime, there have been significant advancements in analytical methodologies used for dioxin analysis (Reiner et al., 2006). The regulatory bodies have recommended the use of tandem mass spectrometry as an alternative confirmatory method, provided that the same method undergoes rigorous validation in accordance with stringent regulatory standards (USEPA, 2022; European Union, 2017). To meet these regulatory requirements, it is crucial to develop advanced clean-up protocols that can effectively remove co-extracts from the crude extract during dioxin analysis. The classical approach, which utilizes multilayer silica, alumina, and activated carbon (AC) based column chromatography, remains irreplaceable due to its applicability in wide range of matrices. This method effectively eliminates co-extracts and separates analytes of interest into distinct fractions based on their planarity. The ready-to-use commercially available cartridges packed with the above mentioned sorbents, particularly those based on AC, come at a relatively high cost. This expense considerably raises the analysis costs for dioxins and PCBs, ultimately limiting the regular monitoring of these contaminants.

The primary goal of this study is to produce cost-effective AC derived from coconut shells using chemical activation method, which can be effectively employed in dioxin sample preparation. Coconut shells, which are typically discarded as biomass waste in regions like south India, Indonesia, Philippines, etc., have garnered attention as a promising resource for synthesizing AC (Hao et al., 2018; Keppetipola et al., 2021; Kim et al., 2022; Piriya et al., 2021; Zulkefli et al., 2022). By utilizing coconut shells as a raw material for AC production, this study seeks to achieve two significant advantages: the valorization of biomass waste and the generation of a valuable sorbent material. Carbon can undergo chemical activation by various activating agents including acids, bases, and salts (Azam et al., 2022; Chen et al., 2022; Hao et al., 2018; Heidarinejad et al., 2020; Neme et al., 2022). These agents play a crucial role in the activation process, leading to the development of unique AC materials with diverse properties and applications. There is literature evidence stating the effective use of AC for mitigating organic pollutants like polycyclic aromatic hydrocarbons (PAHs) through adsorption (Mirzaee and Sartaj, 2022; Hoc Thang et al., 2021; Zhang et al., 2010; Heidarinejad et al., 2020; Yang et al., 2018; Haghseresht et al., 2002). Present study reports effective use of potassium hydroxide (KOH) based chemical activation to obtain AC with desired properties, making it fit for dioxin sample preparation.

After successfully synthesizing the AC, a comprehensive characterization process was undertaken, which included scanning electron microscope (SEM) analysis and N_2 adsorption-desorption experiments. These procedures were conducted to explore and comprehend the surface characteristics and textural properties of the AC material. While the majority of existing studies (Chi et al., 2006; Hung et al., 2011; Long and Yang, 2001; Tu et al., 2021; Zhan et al., 2022) focus on utilizing activated carbon based adsorption to mitigate dioxins and PCBs, there is a lack of comprehensive research investigating the distinct adsorption characteristics of individual congeners within this class of compounds onto activated carbon. To address this research gap, the present study conducted batch adsorption experiments with 21 compounds, comprising 17 dioxins and 4 coplanar non ortho (NO) PCBs, using the synthesized AC as the adsorbent. The obtained adsorption data was further analyzed to identify the most suitable kinetic and adsorption isotherm models that effectively describe the adsorption process of these pollutants. By filling this knowledge void, the study aims to contribute valuable insights into the behavior of these compounds when interacting with AC, which could have significant implications in analytical applications.

In the subsequent phase of the investigation, the potential of converting the synthesized activated carbon (AC) into a highly efficient sorbent was explored to aid in both fish oil decontamination and dioxin sample preparation (**Chapter 5**). The AC sorbent was utilized in fish oil decontamination trials, with variations in the activated carbon load. Throughout this phase, meticulous monitoring of fish oil quality parameters ensured that no deterioration occurred during the decontamination process. Overall, this innovative approach presents new opportunities for regular, cost-effective decontamination of dioxins and PCBs. Its potential applications span various analytical settings and regulatory frameworks, ultimately contributing to enhanced food safety

4.3. Materials and methods

4.3.1. Chemicals and consumables

The carbon source employed was coconut shells, which were obtained from local source. Analytical grade KOH, Na_2CO_3, H_2SO_4, were obtained from Merck-India, certified reference standards of both native and ^{13}C labeled dioxins and PCBs were procured from Cambridge

Isotopic Laboratories (CIL), Massachusetts, USA. High purity headspace GC grade solvents, including methanol, hexane, toluene (Spectrochem, India), and n-nonane (E-Merck, Germany), were used for various experiments. The deionized ultrapure water, employed for cleaning the coconut shell and washing off KOH, was obtained from a Milli-Q Ultrapure water purification system (Millipore, Brussels, Belgium). For decontamination trials with the prepared AC, feed grade fish oil samples were obtained from the local market and utilized as representative matrix. Multilayer silica, carbon and alumina cartridges were procured from Fluid Management Systems (FMS Inc., Waltham, MA, USA). Helium and nitrogen gas (99.999%, Bhoruka, India) were employed as carrier and collision gasses, respectively, in the GC-MS/MS analysis.

4.3.2. Preparation of KOH-AC

The coconut shells obtained were thoroughly cleaned with deionized water to remove impurities. Coconut shells were heated to 110°C overnight and ground into powder. The obtained carbonaceous powder was sieved with a 125 µm sieve and used for chemical activation. The impregnation ratio of AC to KOH (AC: KOH) was 1:3. The obtained carbonaceous powder was mixed with calculated weight of KOH to maintain the impregnation ratio. The mixture was dispersed in deionized water and stirred for 4 hours. After stirring, the mixture was heated in a hot air oven at 110°C until dry. The dried powder was washed with deionized water to remove KOH. Complete removal of KOH from AC was ensured by monitoring pH at regular intervals. The final thermal activation of the prepared material was carried out at 800°C in a tubular furnace with nitrogen atmosphere. After calcination the material was allowed to cool and transferred into a glass bottle and stored in a desiccator.

4.3.3. Characterization of KOH- AC

To examine the surface morphology characteristics of the AC, a scanning electron microscope (Zeiss E-SEM microscope) operating at 15/20 keV was utilized. In order to assess important factors related to adsorption capacity, such as specific surface area, pore size distribution, and pore volume, a N_2 sorption experiment was carried out using a physisorption analyzer (Sync 400, Altamira Instruments, Inc, USA). The surface area was determined based on the Brunauer-Emmett-Teller (BET) theory, while the Barrett–Joyner–Halenda (BJH) method was employed to estimate the pore size distribution. Before the analysis, the sample was degassed under vacuum

conditions at 300°C for 16 hours. Subsequently, the sample was cooled using liquid nitrogen while maintaining a bath temperature of 77.35 K, enabling the acquisition of nitrogen sorption isotherms. This involved sequentially introducing and removing known volumes of nitrogen while simultaneously measuring the equilibrium pressure. The BET surface area measurement was conducted at relative pressure (P/P0) equal to 0.2 while pore volume estimation was done at P/P0 equal to 0.99.

4.3.4. Batch adsorption and kinetic experiments

Adsorption experiments are pivotal in evaluating the efficacy of the prepared AC for adsorbing dioxins and NO-PCBs from a non-polar solvent medium. In contrast to the conventional use of hexane as the non-polar elution solvent for dioxins and PCBs sample preparation, this study diverges by employing n-nonane as the medium for both adsorption and kinetic batch experiments. This choice is based on the comparable polarity of n-nonane to n-hexane and its higher boiling point, ensuring suitability for the experimental procedures. For the batch adsorption studies, analyte solutions were prepared by diluting stock concentrations of native dioxins and NO-PCBs in n-nonane solvent. The dioxins mixed standard solution followed the concentration ratio of TCDD/Fs: (Pe,Hx,Hp)CDD/Fs: OCDD/Fs as 1:2.5:5. Six different initial concentrations of the analytes, ranging from 750 pg/ml to 2000 pg/ml (in terms of TCDD/Fs and NO-PCBs), were prepared for the adsorption isotherm experiments. In each experiment, 1 ml of the prepared analyte solution was mixed with the optimized AC load (1 mg) and stirred until reaching the equilibrium time determined from the kinetic experiments. Then, 10 µl of the supernatant solution was transferred to a gas chromatograph (GC) vial equipped with a micro insert for analysis. The residual concentrations of analytes were determined using GC coupled with tandem mass spectrometer (GC-MS/MS). The same procedure was employed for the kinetic experiments, with the optimized concentration of analyte (obtained from the adsorption studies). The concentration of analytes was monitored at different time intervals (0-135 minutes) to investigate the kinetic behavior of the adsorption process.

4.3.5. Adsorption isotherms

The adsorption isotherms were employed to investigate the adsorption mechanism of dioxins and PCBs and optimize the use of AC. In this study, we correlated the experimental data from

isotherm experiments using three models: Freundlich, Langmuir, and Temkin isotherm models. The primary parameter used to construct the various isotherms was the adsorption capacity of the adsorbent at equilibrium condition, represented as q_e. Its calculation is based on the following equation:

$$q_e = \frac{(C_i - C_e)}{W} \times V \text{ ------} (\textbf{Eq 4.1})$$

Where C_i is the initial concentration of the analyte and C_e is the equilibrium concentration of the analyte. According to the Freundlich adsorption isotherm model, it is assumed that the adsorbent surface possesses a multitude of adsorption sites with different sorption capacities. This results in a heterogeneous adsorption process with the formation of multiple layers. The interaction between the adsorbent and the adsorbate is predominantly governed by physical forces, such as Van der Waals interactions. The Langmuir adsorption isotherm model operates under the assumption that the adsorbent possesses a limited quantity of uniform binding sites, each with equal affinity for the adsorbate molecules. Each binding site can accommodate only one adsorbate molecule, resulting in monolayer adsorption, which occurs through the formation of chemical interactions. Since present study carried out adsorption experiments with all the analytes (PCDD/Fs and NO PCBs) together we have employed the Temkin adsorption isotherm model also which considers adsorbate-adsorbate interactions. The Temkin adsorption isotherm also takes into account that the heat of adsorption decreases linearly as the adsorbate coverage on the surface increases, resulting in weaker attractive forces between the adsorbate and the surface as the surface becomes more densely covered with adsorbate molecules. Using the obtained adsorption experimental data, we plotted different isotherms by employing linear equations corresponding to each model for each analyte as described in the **Table 4.1.** The best fit in case of each analyte was selected considering the regression coefficient (R^2) which indicates how well the model fits the experimental data.

Table 4.1

Adsorption and kinetic model equations

Adsorption studies		Kinetic studies	
Model	Equation (linear form)	Model	Equation (linear form)
Freundlich	$\ln q_e = \ln K_f + \frac{1}{n} \ln c_e$….(**Eq 4.2**)	Pseudo first order	$\ln(q_e - q_t) = \ln q_e - K_1 t$....(**Eq 4.5**)
Langmuir	$\frac{1}{q_e} = \frac{1}{K_L\, q_{max}} \times \frac{1}{c_e} + \frac{1}{q_{max}}$…(**Eq 4.3**)	Pseudo second order	$\frac{t}{q_t} = \frac{1}{q_e} \times\ t + \frac{1}{K_2 * {q_e}^2}$…….(**Eq 4.6**)
Temkin	$q_e = B \ln A_T + B \ln c_e$….(**Eq 4.4**)	Intra particle diffusion	$q_t =\ K_3 t^{\frac{1}{2}} + A$…….(**Eq 4.7**)
q_e = adsorption capacity of adsorbent, c_e = equilibrium concentration of adsorbate, 1/n = intensity of adsorption /degree of curvature, K_f = Freundlich Partition Coefficient, K_L = Langmuir affinity constant, B = constant related to heat of sorption (J/mol), A_T = Temkin isotherm equilibrium binding constant.		q_t and q_e are the amount of adsorbate adsorbed at a given time t and at equilibrium time. K_1 and K_2 are rate constants, K_3 is the diffusion constant and t is the time.	

4.3.6. Kinetic models

To enhance the comprehension of adsorption dynamics of the analytes and to optimize the adsorption process for practical applications, three kinetic models were employed: the pseudo-first order (PFO), pseudo-second order (PSO), and intra-particle diffusion (IPD) models. The PFO model is appropriate when the concentration of the adsorbent significantly surpasses that of the adsorbate. It is based on the assumption that the uptake rate of solute is directly proportional to the difference between the saturation concentration and the amount of solid uptake over time. On the contrary, the PSO model assumes that the rate-limiting step is chemical sorption, providing predictions across the entire adsorption range. It considers the adsorption rate to be dependent on the adsorption capacity rather than the concentration of the adsorbate. Additionally, alongside the aforementioned models, the IPD model was employed to examine the rate-controlling step of the adsorption process. This model assumes that adsorption occurs in multiple steps, with IPD being one of the potential rate-limiting steps. While processing the kinetic data, the significant parameter q_t, adsorption capacity of the adsorbent at a particular time t, is also calculated as per **Eq 4.1**, where concentration of analyte at equilibrium (C_e) is replaced

by concentration of analyte at time 't' (C_t). Obtained Kinetic data points were fitted into linear forms of these kinetic models (**Table 4.1.**) and evaluated various parameters. The most appropriate model was selected based on the R^2 values obtained for each congener.

4.3.7. Sample preparation and quantification of analytes

Dioxins and PCBs quantification utilized a GC-MS/MS system featuring an Agilent Technologies 7890B gas chromatograph equipped with a 7690A automatic liquid sampler and a 7000C triple quadruple mass spectrometer. The developed GC-MS/MS confirmatory method (**Chapter 2**) was employed to quantify analytes in both batch adsorption experiments and fish oil decontamination trials. As the fish oil matrix is 100% fat, the 2 g sample underwent direct clean-up without extraction. Conventional multilayer silica, alumina, and carbon cartridges were used for clean-up, arranged in the order of multilayer silica-carbon-alumina. The entire setup was connected to a vacuum pump for vacuum-assisted solvent elution. The column assembly was conditioned with n-hexane solvent, followed by the addition of the sample in 60 ml of hexane along with 13C12 labeled internal standards for all analytes. The assembly was then eluted with an optimized volume of n-hexane. Subsequently, analytes trapped in the carbon and alumina columns were reverse eluted using toluene. The resulting fractions were concentrated using a nitrogen evaporator and introduced into the GC-MS/MS system for quantification

4.3.8. Fish oil decontamination studies

The synthesized activated carbon was employed for fish oil decontamination experiments. Contaminant-free fish oil, confirmed through prior analysis showing all congeners below the designated LOQ value, was utilized. The fish oil was spiked with the maximum allowed level (ML spiking) of PCDD/Fs & NO-PCBs. Decontamination trials were conducted with varying loads of activated carbon. Three different experimental trials were carried out by adjusting the weight ratio of fish oil to activated carbon (Fish oil: AC; 1:0.01, 1:0.05, and 1:0.1). In a typical decontamination trial, 2g of fortified fish oil was combined with activated carbon according to the specified weight ratio. The mixture was stirred using a magnetic stirrer for 2 hours, representing the optimized equilibration time. After equilibration, the mixture was centrifuged at 2000 rpm for 2 minutes, and the supernatant fish oil (free of activated carbon) was recovered and

divided into two portions. One portion was subjected to quantification of analytes using GC-MS/MS, while the other portion was analyzed for fish oil quality parameters.

4.3.9. Estimation of fish oil quality parameters

To ensure the preservation of fish oil quality following the activated carbon-based decontamination process, three key quality parameters were carefully monitored: the fatty acid profile, free fatty acid value, and acid value. This rigorous monitoring aimed to assess the effectiveness of the decontamination process in maintaining the integrity and nutritional value of the fish oil. The detailed methodology employed to monitor these parameters is outlined below.

- Fatty acid profiling (Fatty Acid Methyl Ester, FAME method)

The Fatty Acid Methyl Ester (FAME) method is employed to profile fatty acids in fish oil samples. Initially, fatty acids undergo derivatization via transesterification with an acid catalyst and methanol. Typically, 0.5 g of fish oil is mixed with 1 ml of 2% methanolic sulfuric acid and refluxed for 5 to 6 hours at 55°C. Thin Layer Chromatography (TLC) verifies the completeness of derivatization. After cooling, the oil is washed two to three times with 2 ml of hexane, followed by a similar wash with 2% sodium bicarbonate solution and distilled water. These washes are combined in a separating funnel. After agitation, the top organic layer, containing derivatized fatty acids, is collected, while the bottom aqueous layer is discarded. The collected fraction is then filtered through a sodium sulfate-containing syringe filter. Subsequently, the filtered derivatized fraction undergoes Gas Chromatography with Flame Ionization Detection (GC-FID) analysis. The acquired data is processed using previously obtained instrument calibration data, which involves injecting a certified mixed fatty acid standard. This process determines the fatty acid profile of the sample.

- Free Fatty Acid (FFA), Acid value

To prepare the sample, 2g of the fish oil sample is mixed with 12 mL of methanol and heated until bubbles begin to appear. A drop of phenolphthalein indicator is then added, and the mixture is titrated against 0.1 N standardized KOH solution. Equation x and y are utilized to calculate the Free Fatty Acid (FFA) content and the Acid Value of the sample.

$$FFA\ value = \frac{M \times N \times V}{10 \times W} \text{.....(4.8)}$$

$$Acid\ value = \frac{56.1 \times N \times V}{W} \text{.....(4.9)}$$

M= molecular weight of main triglyceride fraction, N = normality of KOH solution, V = volume of KOH, W = weight of the sample)

4.4. Results and discussions

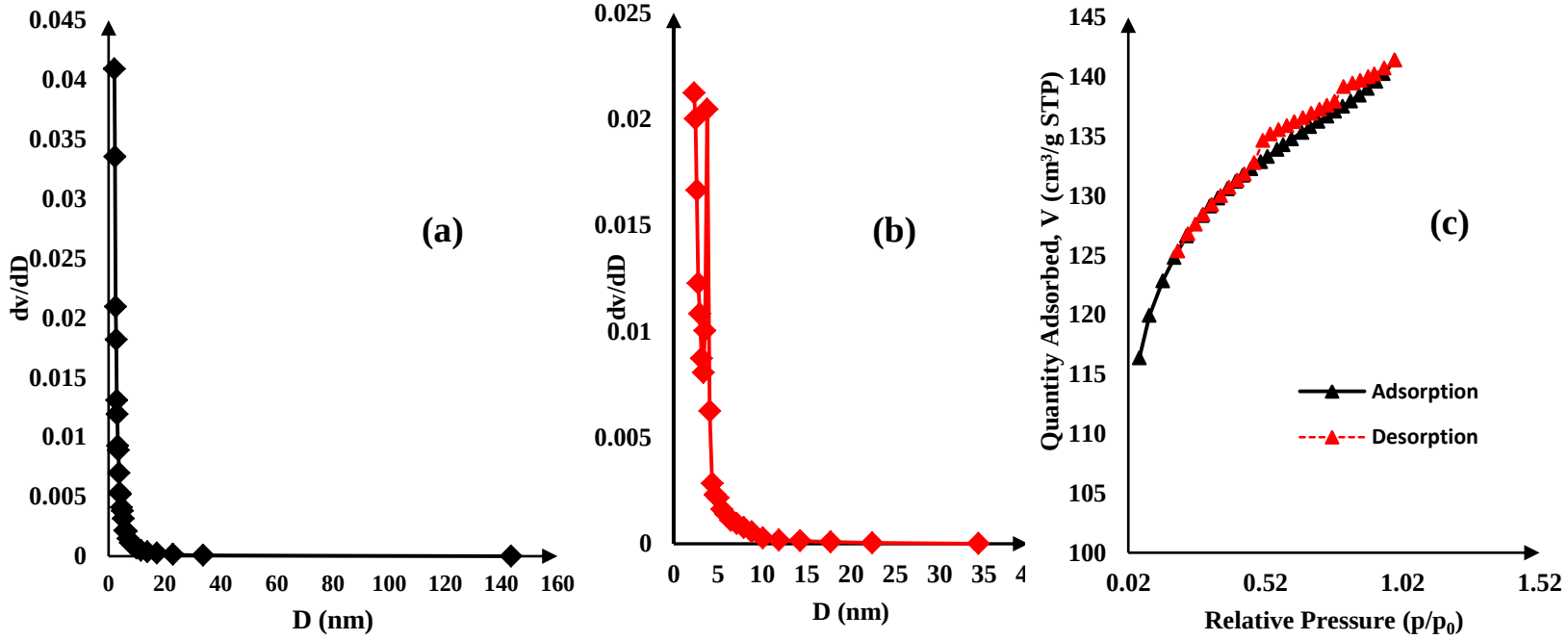
4.4.1. Morphology characteristics of AC

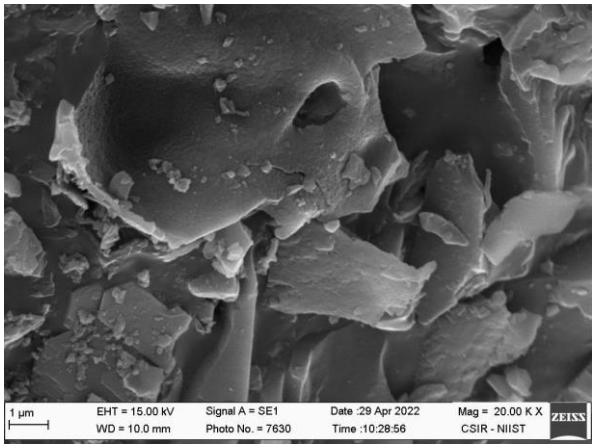
The SEM images (Fig. 4(d) and 4(e)) of the prepared AC were captured before and after chemical activation, revealing distinct observations regarding the formation of pores. Carbon before activation exhibited a rough surface devoid of any visible pores, while after activation it displayed clear pore formation. This transformation indicated a significant structural change induced by the chemical activation process. There is literature evidence stating increased KOH concentration during activation process enhances the surface area significantly by the formation of pores in the surface (Li et al., 2017; Nandi et al., 2023). The presence of visible pores suggested that the high concentration of KOH used during the activation process is in line with the earlier observations. Importantly, the absence of clogged pores indicated that the AC maintained its structural integrity while achieving pore formation. This enhancement in porosity translates to an increased surface area, enabling improved adsorption capacity and efficiency during the sample preparation step of dioxin analysis. These findings support the possibility to use of chemically activated carbon for fabricating ready-to-use AC cartridges, offering enhanced accessibility and availability of active sites for analytes.

4.4.2. Textural characteristics of AC through N_2 sorption experiments

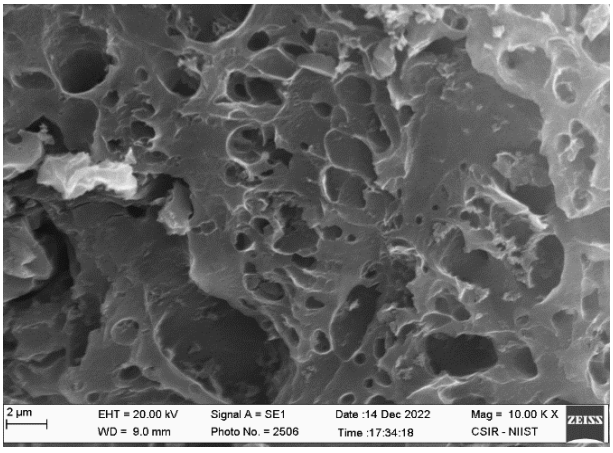
Fig. 4.1 (a)-(c) and **Table 4.2** present the textural characteristics of the prepared AC, analyzed through N_2 adsorption-desorption studies using the BET and BJH methods. The obtained N_2 adsorption-desorption isotherm aligns with the typical IUPAC Type IV class, consistent with prior studies on chemically AC from various carbon sources (Zhao et al., 2022). This observation confirms the mesoporous nature of the prepared material and its multilayer adsorption behavior. The BJH method revealed a median pore diameter of 2.26 nm, further confirms the presence of mesopores, known for their high adsorption selectivity towards analytes, making them advantageous for applications requiring the removal or separation of specific target molecules from mixtures. The AC exhibits a high BET surface area of 390.01 m^2/g, signifying a substantial contact area available for the adsorption of target species. This feature is highly desirable for

adsorbents as it suggests efficient utilization of the material's surface for adsorption purposes. Additionally, the measured pore volume of 0.219 cm^3/g highlights the abundance of pores within the AC, providing increased adsorption sites and enhancing adsorption capacity. The accessibility of these pores is vital for efficient adsorption, facilitating easy diffusion of adsorbate molecules into the material's internal structure. Table 1 offers a comparison of the textural characteristics of coconut shell carbon before and after chemical activation. The results demonstrate a significant increase in the surface area (25 times) and pore volume (15 times) while maintaining the structural integrity. Overall, the findings, encompassing the high BET surface area, substantial pore volume and presence of mesopores, indicate that the prepared AC possesses favorable characteristics for adsorbing dioxins and PCBs during sample preparation for dioxin analysis of complex matrices.





(d)



(e)

Fig.4.1. Textural properties of prepared AC **1(a)** Pore size distribution **1(b)** desorption pore size distribution **1(c)** N_2 adsorption-desorption isotherm plots and SEM images of AC 1(d) Before activation 1(e) Activated using KOH

Table 4.2

Textural properties comparison before and after chemical activation process of coconut shell derived carbon

SI No	Parameter	Unit	Value	
			Before activation	After activation
	Surface Area			
1	Single Point Surface Area at p/po=0.20000	m^2/g	14.9	436.5
2	BET Surface Area		14.6	390.0
3	BJH Adsorption Cumulative Surface Area		11.2	65.3
4	BJH Desorption Cumulative Surface Area		10.6	48.7
	Pore Volume			
5	Total Pore Volume at $p/p^0 = 0.99000$	cm^3/g	0.016	0.219
6	BJH Adsorption Cumulative Pore Volume		0.014	0.055
7	BJH Desorption Cumulative Pore Volume		0.013	0.044
	Pore Size			
8	Average Pore Diameter (4V/A):	nm	4.37	2.24
9	BJH Adsorption Medain Pore Diameter:		5.52	2.97
10	BJH Adsorption Most Frequent Pore Diameter (dV/dD)		2.06	2.06
11	BJH Desorption Medain Pore Diameter		4.12	3.35
12	BJH Desorption Most Frequent Pore Diameter (dV/dD)		3.80	2.31

4.4.3. Adsorption isotherms

The outcomes of the batch adsorption experiment were subject to thorough analysis using linear fitting techniques, employing three well established adsorption isotherm models: The Freundlich, Langmuir and the Temkin model. **Table 4.3** presents diverse parameters derived from the linear plots, along with the ranking of the examined adsorption models while the **Fig.4.3.** depict the characteristic isotherms obtained for the most toxic congener, 2378-TCDD. By assessing the R^2 values of each model, it became evident that the adsorption experimental data exhibited a strong alignment with the sequence of isotherm models in the order: Langmuir > Freundlich > Temkin.

When considering the batch adsorption outcomes for all congeners collectively, it became apparent that dioxins exhibited a superior fit across the various models compared to non-ortho PCBs. This phenomenon can be attributed to the inherent planarity of the congeners. While both dioxins and NO-PCBs possess planar qualities, NO-PCBs display a slightly lower degree of rigidity in their planar structure compared to dioxins. This distinction is significant because the adsorption of aromatic compounds onto AC is predominantly driven by π-πstacking interactions (**Fig. 4.2**), in which planar delocalized π electrons of aromatic rings interact strongly with the π-electron clouds present in the carbon atoms of the AC surface, which significantly increases the adsorption process. Focusing on the Langmuir adsorption isotherm, the obtained qmax value – indicating the maximum adsorption capacity of the adsorbent ranged from 1265.7 to 37850.5 pg/g. Among analyzed compounds NO-PCBs showed lowest q_{max} value indicating less affinity towards the adsorbent, which is in accordance with the obtained regression values. The values obtained for both PCDDs and PCDFs were consistent with extend of chlorination. Considering the congener groups of dioxins average qmax values were 36100.8 (tetra chlorinated), 12933.7 (penta chlorinated), 5784.1 (hexa chlorinated), 10219.0 (hepta chloritaed) and 17920.0 pg/g (octa chlorinated). The higher qmax values observed for both OCDD/F could potentially be attributed to their elevated concentration (which was five times greater than that of the tetra-chlorinated congeners) within the batch adsorption experiment mixture. It is noteworthy that compounds with higher molecular weights (indicating greater chlorine substitution) typically display lower qmax values, thus further reinforcing the influential role of planarity in dictating the extent of adsorption. The "R_L" value referred as the separation factor or Langmuir adsorption equilibrium parameter serves as a predictive tool for understanding the adsorption behavior on a surface. It aids in assessing whether the adsorption process leans towards being favorable or unfavorable. In the present study the obtained R_L values of individual compounds ranged from 1.36×10^{-7} to 3.60×10^{-6}, obtained low values ($R_L < 1$) indicates that studied adsorption process is favorable and efficient according to the Langmuir isotherm model. Considering the other two adsorption models Freundlich model shown reasonably good fit to experimental data for all the analyzed compounds. The obtained 1/n values fall in the range "0<1/n<1", indicating the adsorption process is highly favorable and there exist a stronger interaction between adsorbate molecules and adsorbent. In line with both Langmuir and Freundlich model Temkin model also gave reasonably good fit to experimental data. The K_T values obtained displayed uniformity across

individual compounds, suggesting a homogeneous AC surface and consistent interaction strength between each compound and the adsorbent.

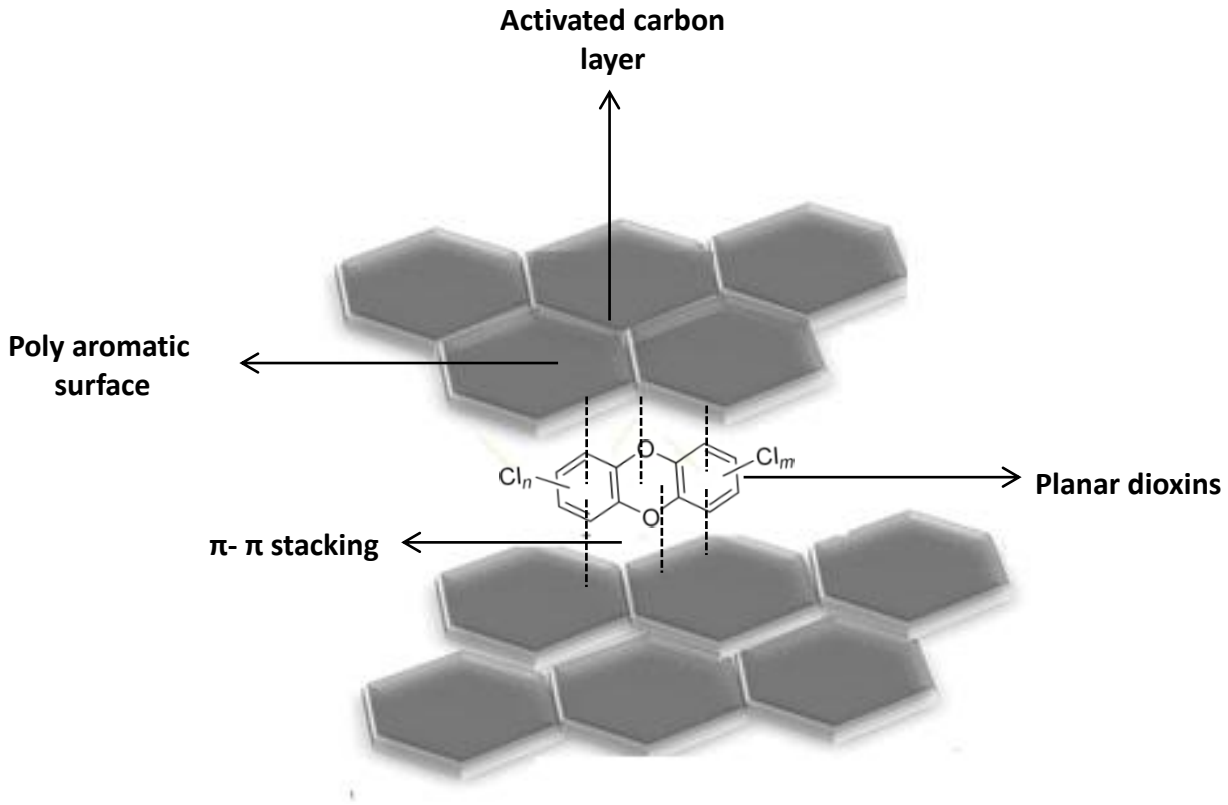


Fig.4.2 Dioxins trapped in AC layer through π- π stacking (Illustrates the entrapment of dioxins within the activated carbon (AC) layer facilitated by π-π stacking interactions).

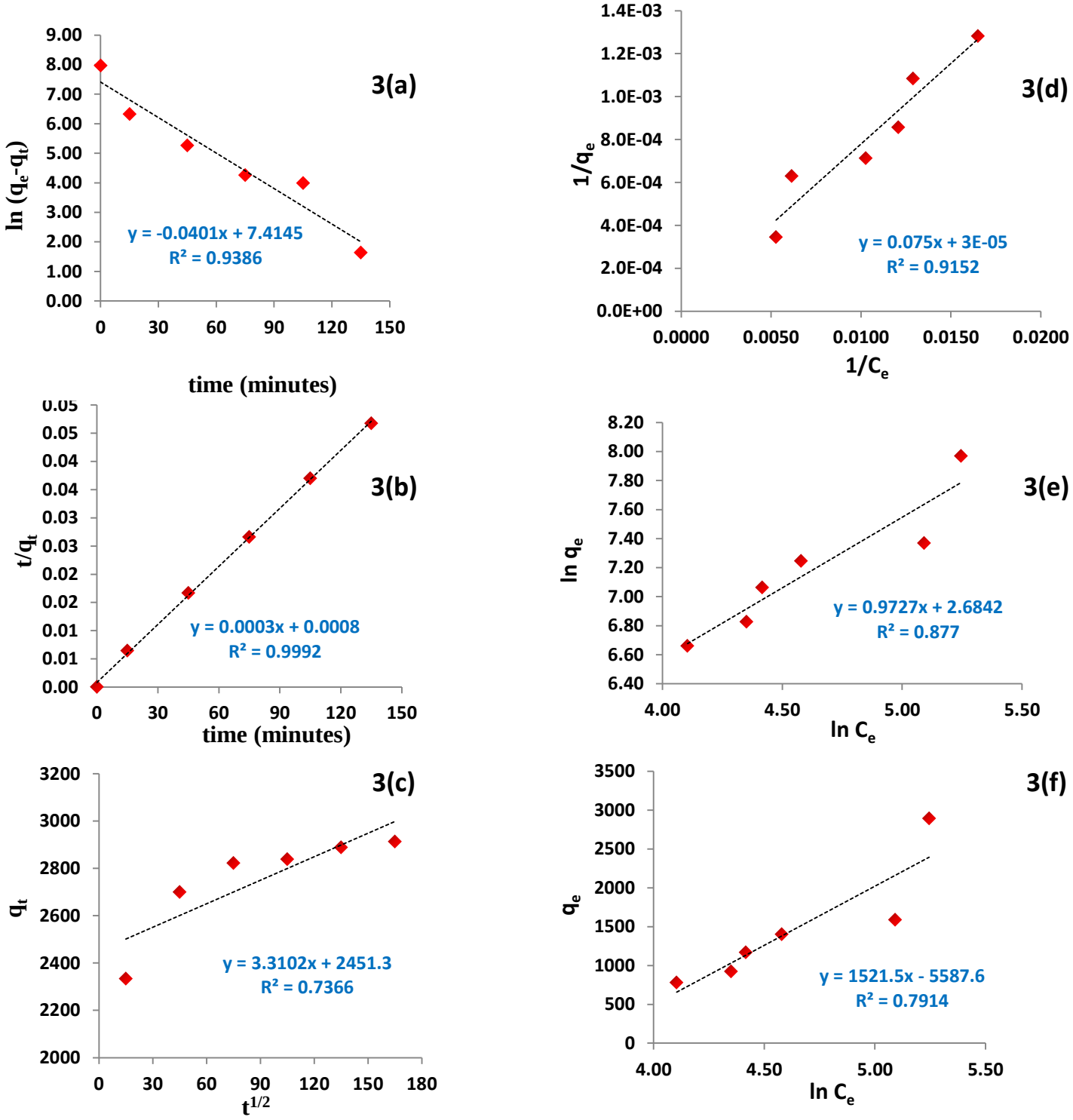


Fig 4.3. Representative linear plots of different kinetic and adsorption isotherm models obtained for the congener 2378-TCDD **3(a)** PFO, **3(b)** PSO, **3(c)** IPD **3(d)** Langmuir, **3(e)** Freundlich, **3(f)** Temkin

4.4.4 Kinetic modeling

The adsorption kinetics of dioxins and PCBs into prepared AC was analyzed and interpreted using three kinetic models: PFO, PSO and IPD. The regression coefficient analysis provided insights into the adequacy of each model in describing the experimental data. **Table 4.3** visually represents the order of these kinetic models, illustrating their effectiveness through regression coefficient values. Additionally, it provides a structured overview of the parameters extracted from the models, offering a comprehensive understanding and the **Fig 4.3.** Supplement this information by displaying unique linear plots that showcase different kinetic models, with a specific focus on the congener 2378-TCDD. The analysis of the adsorption batch data revealed that most of the congeners exhibited the best fit when fitted into different kinetic models in the following order: PSO > PFO > IPD. Evaluation of rate constant value of the most suited kinetic model, PSO shown decrease in adsorption rate as molecular weight of the congener increases. It can be attributed to the fact that planarity of the congener is inversely proportional to the no of chlorine substitutions in the aromatic rings. Notably, the findings from the observed data further support the π-π stacking phenomenon, which is highly significant in the adsorption of dioxins and PCBs onto AC. The obtained regression coefficient values for the PFO model (ranging from 0.557 to 0.981) suggest that it may not fully represent the adsorption kinetics for all congeners as effectively as the PSO model. While it still provides reasonable fits for most of the congeners, its overall adequacy is lower compared to the PSO model. Considering the IPD model, the regression coefficient values were less for low molecular weight bearing compounds. However, the experimental data for higher molecular weight bearing compounds showed a good fit to the IPD model. The IPD model's ability to adequately represent the adsorption process seems to improve as the molecular weight of the congeners increases. This observation further strengthens the lack of the π-π Stacking as molecular weight increases. Hence, it may be inferred that higher chlorinated compounds tend to adsorb through diffusion process into the available meso/micro pores of the AC surface.

Table 4.3 Obtained adsorption isotherm, kinetic model parameters and regression coefficient ranking corresponding to individual congeners

Compounds	Adsorption isotherm models										Kinetic models								
	Langmuir			Freundlich				Temkin			PFO			PSO			IPD		
	K_L	q_{max}	R^2	R_L	K_F	1/n	R^2	B	K_T	R^2	q_e	K_1	R^2	q_e	K_2	R^2	A	K_3	R^2
PCB 81	498.91	1500.60	**0.69**	2.20E-06	27.41	0.53	**0.62**	423.10	0.01	**0.54**	480.8	0.02	**0.93**	607.31	1.61E-04	**0.98**	311.00	2.25	**0.90**
PCB 77	601.63	2673.21	**0.85**	1.66E-06	21.63	0.65	**0.82**	630.21	0.02	**0.73**	513.00	0.02	**0.88**	788.94	2.01E-04	**0.99**	494.31	2.33	**0.80**
PCB 126	464.82	1265.73	**0.74**	2.42E-06	29.41	0.50	**0.70**	366.72	0.01	**0.63**	452.41	0.02	**0.94**	532.85	1.59E-04	**0.98**	251.52	2.11	**0.91**
PCB 169	278.12	1612.82	**0.88**	3.60E-06	53.32	0.48	**0.87**	423.90	0.02	**0.82**	655.22	0.00	**0.56**	379.00	3.07E-04	**0.88**	208.90	1.48	**0.57**
2378-TCDF	2906.92	37850.52	**0.93**	3.13E-07	22.21	0.87	**0.91**	1053.43	0.04	**0.94**	805.12	0.02	**0.92**	1380.91	1.73E-04	**0.99**	1022.40	2.81	**0.90**
2378-TCDD	2575.92	34351.23	**0.92**	3.88E-07	14.64	0.97	**0.88**	1521.50	0.03	**0.79**	1659.92	0.04	**0.94**	2914.41	1.49E-04	**1.00**	2404.81	4.16	**0.74**
12378-PeCDF	1196.80	14802.82	**0.90**	3.85E-07	39.23	0.76	**0.87**	2349.50	0.01	**0.81**	1716.31	0.02	**0.94**	2658.00	7.20E-05	**1.00**	1814.41	6.63	**0.83**
23478-PeCDF	1120.40	10252.12	**0.91**	5.12E-07	21.14	0.81	**0.89**	2317.23	0.01	**0.77**	1027.11	0.03	**0.96**	1685.40	1.41E-04	**1.00**	1215.12	3.76	**0.76**
12378-PeCDD	921.83	13746.23	**0.94**	4.86E-07	39.65	0.78	**0.90**	2437.01	0.01	**0.81**	1800.32	0.03	**0.95**	2725.12	8.62E-05	**1.00**	1984.00	5.86	**0.81**
123478-HxCDF	342.71	4655.00	**0.92**	1.20E-06	197.42	0.42	**0.89**	1080.04	0.03	**0.87**	1607.32	0.03	**0.98**	2218.61	7.41E-05	**1.00**	1433.91	6.13	**0.82**
123678-HxCDF	333.22	4988.53	**0.93**	1.23E-06	145.61	0.48	**0.90**	1369.30	0.02	**0.80**	1218.00	0.02	**0.96**	1629.94	7.61E-05	**0.99**	933.42	5.37	**0.85**
234678-HxCDF	445.43	6197.31	**0.89**	1.01E-06	102.60	0.55	**0.85**	1576.42	0.02	**0.77**	1518.21	0.03	**0.98**	2074.61	9.64E-05	**1.00**	1441.43	4.97	**0.82**
123478-HxCDD	397.25	5803.63	**0.92**	1.01E-06	152.10	0.49	**0.88**	1339.20	0.02	**0.87**	2436.72	0.03	**0.94**	2717.82	3.63E-05	**0.98**	1447.00	9.46	**0.94**
123678-HxCDD	369.81	6188.42	**0.96**	1.13E-06	140.62	0.52	**0.94**	1446.30	0.02	**0.94**	1716.23	0.03	**0.93**	2221.43	8.64E-05	**1.00**	1526.91	5.43	**0.83**
123789-HxCDD	397.21	6469.12	**0.94**	1.01E-06	120.9	0.54	**0.96**	1544.94	0.02	**0.97**	1606.12	0.02	**0.93**	2169.41	6.08E-05	**0.98**	1362.10	6.02	**0.99**
123789-HxCDF	298.00	6187.00	**0.88**	1.34E-06	158.2	0.52	**0.91**	1494.80	0.03	**0.93**	1619.71	0.02	**0.92**	2187.32	6.06E-05	**0.99**	1383.11	5.98	**0.99**
1234678-HpCDF	395.61	4540.91	**0.90**	1.05E-06	162.4	0.43	**0.88**	1126.41	0.02	**0.83**	1743.21	0.02	**0.89**	1869.50	6.06E-05	**0.98**	1111.10	5.58	**1.00**
1234678-HpCDD	388.82	7149.10	**0.84**	1.06E-06	128.6	0.55	**0.86**	1590.00	0.02	**0.88**	1537.81	0.02	**0.93**	2200.50	7.19E-05	**0.99**	1488.92	5.32	**0.98**
1234789-HpCDF	1172.23	18967.12	**0.89**	3.49E-07	53.6	0.74	**0.84**	2223.90	0.02	**0.85**	2060.81	0.02	**0.91**	2750.31	4.63E-05	**0.99**	1610.60	8.77	**0.869**
OCDD	659.20	13846.91	**0.93**	3.34E-07	183.4	0.55	**0.9**	3300.63	0.01	**0.87**	2702.00	0.03	**0.91**	3440.34	4.63E-05	**0.99**	2310.10	8.51	**0.965**
OCDF	1658.71	21993.10	**0.93**	1.36E-07	77.1	0.67	**0.92**	4042.71	0.01	**0.92**	2768.00	0.02	**0.93**	3964.00	3.99E-05	**0.99**	2673.50	9.67	**0.99**

*Ranking is based on the highest R^2 value achieved.

Rank 1 **Rank 2** **Rank 3**

4.4.5. Fish oil decontamination studies

Fig. 4.4 presents the results obtained from a series of decontamination trials utilizing synthesized activated carbon, with variations in the fish oil: activated carbon (AC) ratio. Maintaining fish oil: AC ratio of 1:0.01, the decontamination efficiency for dioxins ranged from 46.2% to 62.2%. Notably, the observed decontamination rates were influenced by the planarity of the congeners, with more planar compounds (less chlorinated) demonstrating higher rates, while highly chlorinated, less planar congeners exhibited lower rates. Specifically, the most planar congener, 2,3,7,8-TCDD, exhibited the highest decontamination rate, while OCDD/Fs showed the lowest. This observation underscores the significance of the compound's planarity as a decisive factor in dioxin adsorption onto the AC surface. This finding aligns with previous observations from adsorption experiments, where high q max values were noted for the most planar analytes. Additionally, the observations related to non-ortho PCBs (NO-PCBs) in these decontamination trials revealed lower decontamination rates ranging from 25.2% to 37.43%. This discrepancy in decontamination rates can be attributed to the lower planarity of NO-PCBs compared to dioxins. Furthermore, increasing the AC load in trials with fish oil: AC ratio of 1:0.05 led to improved decontamination rates for all congeners. In these trials, dioxin decontamination rates increased to 60% to 75%, while NO-PCB rates rose from 40% to 60%. Finally, the trial with increased AC content (fish oil: AC 1:0.1) resulted in optimal decontamination of both dioxins and PCBs (90-98%). These findings highlight the crucial role of planarity in determining the extent of adsorption onto activated carbon, particularly through significant π-π stacking interactions between the AC layer and planar aromatic species. The comprehensive nature of these trials provides valuable insights into the factors influencing the decontamination process and underscores the potential of synthesized activated carbon as an effective sorbent for dioxin and PCB removal from fish oil matrices.

4.4.6. Fish oil quality parameter monitoring

The assessment of fish oil quality parameters before and after the decontamination trials offers crucial insights into the effectiveness of the methodology in removing dioxins and PCBs while maintaining the essential qualities of the oil. Fatty acid profiling, a fundamental aspect of this assessment, provides a detailed examination of the types and proportions of fatty acids present in the fish oil. Notably, the analysis revealed a predominant presence of esterified fatty acids,

including Methyl myristate C14, Methyl palmitate C16, Methyl stearate C18, and others, with palmitic acid being the most prominent contributor (**Fig. 4.5**). This comprehensive profiling showcased minimal deviations in the fatty acid profiles before and after the decontamination trials, indicating the stability of essential fatty acid concentrations, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are omega-3 fatty acids with critical health benefits. Furthermore, the evaluation of the Free Fatty Acid Value (FFA) is crucial for assessing the degradation status of the fish oil. The FFA value, ranging from 2.67 to 2.98 with a median of 2.79, demonstrated a negligible change in free fatty acid content post-decontamination. This suggests that the decontamination process had minimal impact on the free fatty acid composition of the fish oil, reaffirming its quality. Similarly, the Acid Value, which measures the concentration of free fatty acids in the oil, exhibited a narrow range of 15.23 to 16.92 (**Fig.4.6**). The minimal variation in the acid value suggests that the decontamination trials had little effect on the overall acidity of the fish oil. Overall, the comprehensive assessment of these quality parameters emphasizes the minimal impact on fish oil quality following the decontamination trials. These findings provide robust evidence of the methodology's efficacy in effectively removing dioxins and PCBs while preserving the essential qualities of the fish oil.

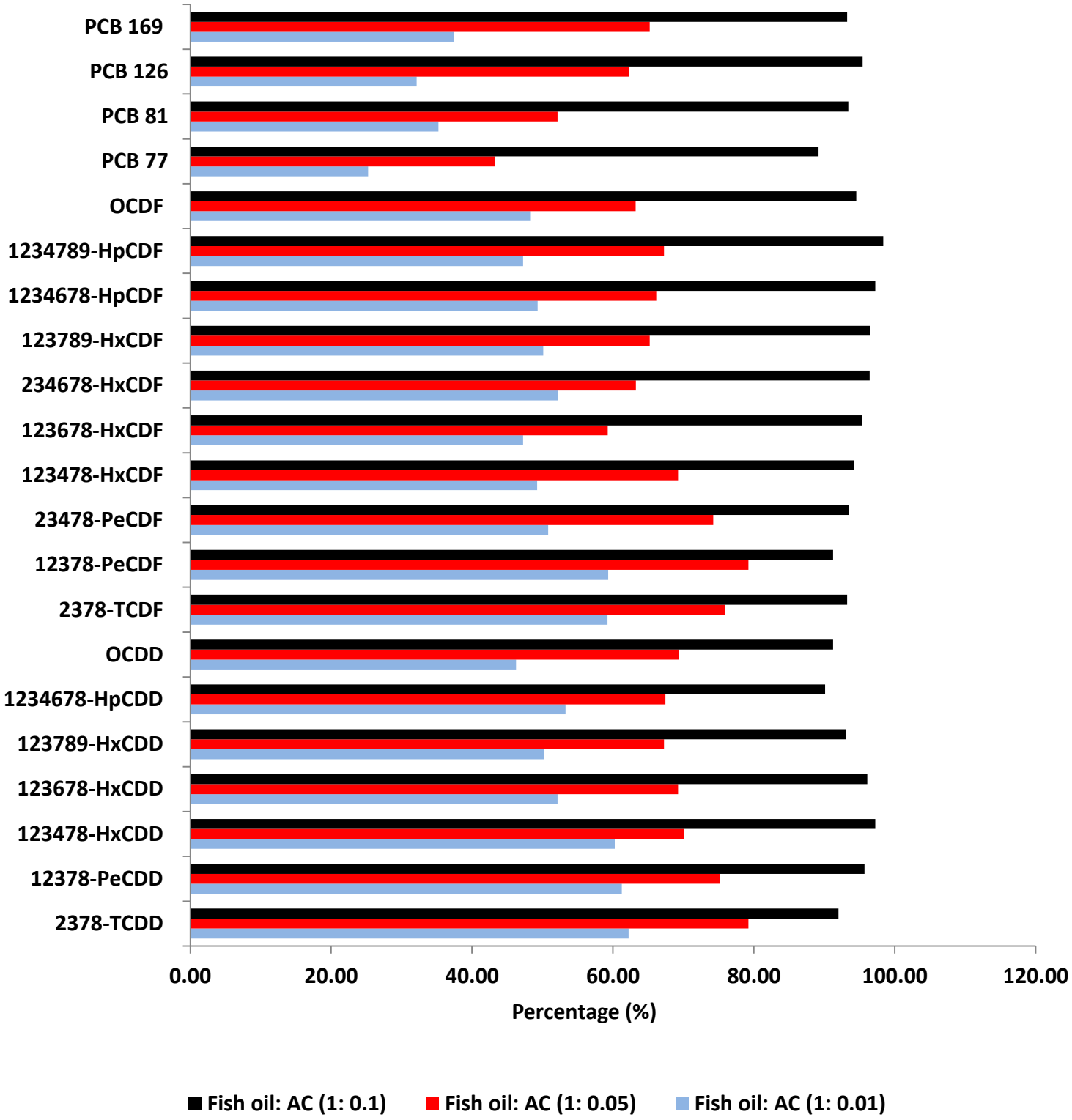


Fig 4.4. Decontamination rate of individual congeners with different AC load

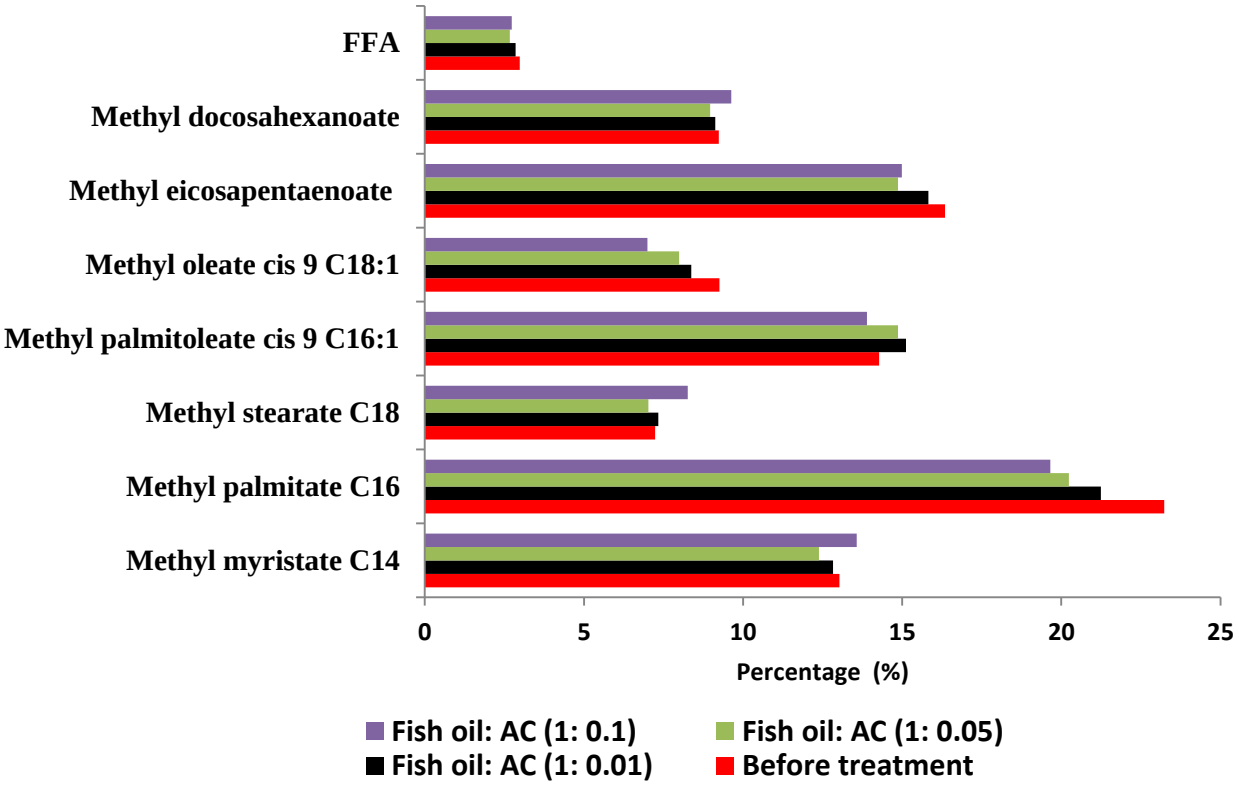


Fig 4.5. Fatty acid profiles before and after decontamination trials

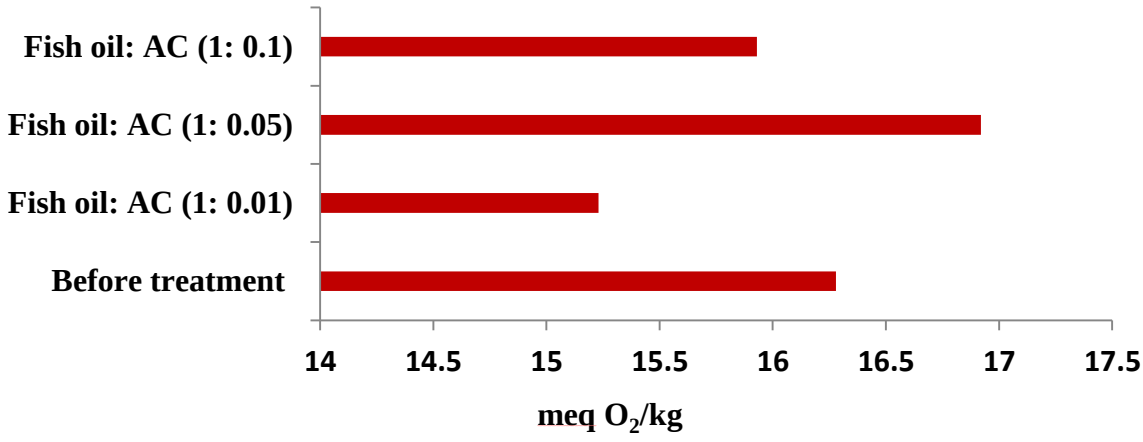


Fig.4.6. Obtained acid values before and after decontamination trials

4.5. Conclusion

The utilization of coconut shell-derived activated carbon (AC) synthesized through a cost-effective chemical activation process with KOH resulted in significant enhancements in surface characteristics and textural properties, boasting a surface area of 390.01 m^2/g and a total pore volume of 0.219 cm^3/g. Batch adsorption experiments involving AC, dioxins, and PCBs showcased a favorable fit, aligning with pseudo-second-order kinetics and the Langmuir isotherm model. Subsequently, the synthesized activated carbon was effectively employed as a cost-effective sorbent to decontaminate dioxins and PCBs from fish oil. Notably, employing an optimized ratio of Fish oil: AC at 1:0.1 yielded impressive removal rates ranging from 90% to 98% for individual congeners. Furthermore, the efficacy of the decontamination strategy was substantiated by the observation of negligible changes in fish quality parameters, including the fatty acid profile, free fatty acid value, and acid value. This comprehensive and cost-effective approach underscores the promising potential of coconut shell-derived activated carbon in facilitating efficient decontamination processes, while preserving the essential qualities of the fish oil.

Chapter

5

Integrated low cost dioxins and PCBs analysis through indigenous clean-up columns: material synthesis, fabrication and method validation

5.1. Abstract

Comprehensive monitoring of dioxins and PCBs in critical food and environmental matrices is essential for safeguarding human health and the environment. However, the high analysis cost has posed a significant challenge to widespread surveillance efforts. Although the acceptance of GC-MS/MS as a confirmatory methodology by regulatory bodies such as the EU has reduced analysis costs, the expense of commercial dioxin/PCBs sample clean-up materials and automated systems remains prohibitive. This study explores the potential for cost reduction through the indigenous synthesis of clean-up materials and the fabrication of cartridges. Various impregnated silica, alumina, and activated carbon materials were successfully synthesized, and a quality control strategy was established. Chromatographic cartridges were fabricated using PTFE-based tubing following numerous optimization trials. Validation of the developed clean-up methodology through spike recovery experiments in fish and fish oil matrices demonstrated high accuracy (within ± 20% bias), optimal ion ratio tolerance (below 15%), and optimum internal standard recovery (within 60-120%). In summary, this research facilitates widespread surveillance of these contaminants by introducing a cost-effective clean-up method to ensure human safety.

5.2. Introduction

The analysis of dioxins/PCBs poses a challenge to analytical chemists due to two critical aspects: the presence of these contaminants at ultra-trace levels and the high analysis cost associated with sophisticated instrumentation (GC-HRMS) and costly sample preparation consumables/automated sample cleanup systems. Since these contaminants are found in complex matrices with numerous co-extracts (such as fats, proteins, phenolic compounds, etc.), the cleanup step in the sample preparation phase is crucial. The sample cleanup must be highly efficient in removing co-extracts and facilitating proper fractionation of analytes to achieve unbiased confirmatory quantification. Generally, laboratories worldwide rely on commercially available materials, but indigenization of sample cleanup can significantly reduce analysis costs (Stefanovic et al., 2019). Despite various sample cleanup analogs available to the scientific community, classical column chromatography employing serially connected multilayer silica, activated carbon, and alumina remains irreplaceable due to its high potency in effectively removing co-extracts and fractionating analytes according to their planarity.

The multilayer silica column consists of various impregnated silica, including 44% H_2SO_4, 2% KOH, and 10% $AgNO_3$ impregnated silica. These impregnated silica materials effectively remove co-extracts through sequential chemical reactions. Acidic treatment of silica with H_2SO_4 modifies the surface chemistry of silica by introducing acidic functional groups such as sulfonic acid and sulfate onto the silica surface. During the introduction of the crude extract, co-extract removal occurs according to the acid-base extraction principle. This technique, commonly used in chemistry intended to separate compounds based on their acid-base properties and solubility in different solvents. The acidic groups present on the silica surface protonates the fatty acids present in the sample and change their polarity from non-polar to highly polar. Since the column assembly is eluted with non-polar n-hexane solvent, the formed polar protonated fatty acid species will stick to the silica column, resulting in the effective removal of fatty acids.

The KOH-treated silica provides a basic environment to facilitate reactions such as trans-esterification, saponification, and aldol condensation. Acidic compounds present in the sample extract, such as carboxylic acids and phenols, can react with the basic site on the KOH-treated silica through acid-base neutralization reactions. The reaction results in the formation of ion pairs/salts, leading to the removal of acidic species from the crude extract. $AgNO_3$-based silica is meant to remove sulfur-containing compounds from the crude extract, such as sulfides and thiols, through silver-sulfur complex formation. The last layer in the multilayer silica column is the sodium sulfate layer, which removes any residual moisture formed during these reactions.

Activated carbon and alumina cartridges play a crucial role in the fractionation of analytes based on their planarity. Dioxins and non-ortho PCBs are planar compounds that can get trapped in the activated carbon cartridge due to its high surface area and pores, facilitating π-π stacking interactions. The alumina cartridge has a great affinity for polar analytes such as mono-ortho and non-dioxin-like PCBs, with the polarity attributed to the electronegativity difference between aluminum and oxygen atoms. Polar PCBs are trapped in this cartridge through dipole-dipole interactions. During the last step of the cleanup phase, activated carbon and alumina cartridges are reverse-eluted to obtain fractions 1 (dioxins and NO PCBs) and 2 (MO and NDL PCBs), respectively.

Recently, various companies have introduced pre-packed, ready-to-use cleanup cartridges of these materials and automated systems that can integrate these columns. Fabricated columns and automated systems play a crucial role in significantly reducing analyte loss during sample preparation. However, despite their versatility, the high cost associated with these cleanup columns and automated systems hinder their widespread acceptance. This fact can be pointed out as the reason for limited surveillance of this class of contaminants, especially in underdeveloped nations or economies in transition. This chapter addresses this fact by developing methodologies for the cost-effective synthesis of cleanup materials and fabrication of them into ready-to-use cartridges. The developed methodology is validated according to EU regulations. This approach can significantly reduce analysis costs and enhance the widespread monitoring of these contaminants.

5.3. Materials and methods

5.3.1. Chemicals and consumables

All reagents (KOH, H_2SO_4, $AgNO_3$) used to prepare clean-up materials were analytical grade and procured from Merck-India. De ionized ultra-pure water was obtained from a Milli-Q Ultrapure water purification system (Millipore, Brussels, Belgium). Head space GC grade high purity solvents procured from Spectrochem, India were used to carry out extraction and clean-up steps, high purity n-nonane(Merck, Germany) was used as keeper solvent during final reconstitution. All certified reference standards used for calibration and isotopic dilution studies were purchased from Cambridge Isotopic Laboratories (CIL), Massachusetts, USA and Wellington Laboratories, Guelph, Canada. It includes Native PCDD/Fs (EDF-5008), Labelled PCDD/Fs (EDF-5430), Native NO PCBs (EC-4986), Native MO PCBs (EC-4987), Native NDL-PCBs (EC-5179), labelled NDL PCBs (EC-4058), Dioxin recovery/syringe standard (EF-5394) and PCB Recovery standard (EC-5371) procured from CIL, while Labelled NO PCBs (MBP-CP) and Labelled MO PCBs (MBP-MO) were from Wellington Laboratories.

5.3.2. Synthesis of clean-up materials

Silica impregnated with 22% and 44% H_2SO_4, 2% KOH, and 10% $AgNO_3$ was prepared. The chromatographic grade silica underwent initial cleaning with methanol and activation at 180°C in a muffle furnace for 4 hours. Overcoming the challenge posed by the deliquescent properties of

H_2SO_4 in achieving free-flowing H_2SO_4-impregnated silica was achieved through a specialized laboratory setup. For impregnation with 2% KOH and 10% $AgNO_3$, predetermined amounts of the reagents were dispersed in water and combined with silica. Subsequent removal of water content was achieved using a rotary evaporator. The activated carbon utilized in this study was derived from coconut shells through chemical activation using KOH followed by thermal activation. A detailed synthesis procedure is provided in section 4.3.2. of chapter 4. The preferred alumina for dioxin/PCBs sample clean-up is basic alumina with activity grade one, which was obtained by purchasing basic alumina and activated thermally to achieve grade I activity.

5.3.3. Fabrication of clean-up materials into chromatographic cartridges and optimization experiments

The prepared clean-up materials were utilized in the fabrication of chromatographic cartridges designed for the sample preparation phase of dioxins and PCBs analysis. A series of trial and error experiments were conducted to optimize various parameters, including material load, packing density, and column sealing. These experiments were meticulously adjusted to achieve the optimal recovery of internal standards within the range of 60-120%. During the fabrication of the AC cartridge, diatomaceous earth was incorporated to enhance its interaction with coplanar analytes. Given the distinct roles of activated carbon (AC) (trapping planar PCDD/Fs, NO-PCBs) and alumina cartridges (trapping MO, NDL-PCBs) in analyte fractionation based on planarity, the efficiency of both was systematically monitored through initial experiments using solvent spiked with internal standards to simulate real sample conditions. Two different activated carbon cartridges with varying filling rates and an alumina column were conditioned with n-hexane (20 ml) and introduced ^{13}C labeled standards of PCDD/Fs -NO PCBs and MO-NDL PCBs respectively in hexane (30 ml) and then further eluted with 220 ml of hexane. Eluted hexane was collected as two fractions, 110 ml each, and named as forward elution fraction 1 and 2 (FEF1, FEF2). Columns were reverse-eluted to obtain Reverse elution fraction (REF), 40 ml toluene. Each fraction was concentrated and injected into GC-MS/MS for quantification. After finishing initial trials, the developed materials were used to conduct real sample analysis in both fish and feed-grade fish oil and carried out method validation as per EU regulation.

5.3.4. Analyzed sample details and quantification of analytes

To assess the effectiveness of the developed methodology, we chose two crucial matrices from the food and feed category: fish and fish oil. Initial analysis of the representative samples revealed negligible levels of analytes, below the limit of quantification, making them suitable for spike recovery experiments. We utilized the optimized extraction procedure outlined in Chapter 2, employing pressurized liquid extraction to extract analytes from the fish matrix. The fish oil samples were directly taken for the cleanup step, bypassing the extraction step. In the initial phase, we analyzed samples spiked with ^{13}C labeled internal standards to optimize the elution volume of the cleanup assembly. This optimization process involved various trial and error experiments aimed at achieving internal standard recovery rates between 60-120%. The resulting analyte fractions were quantified using the GC-MS/MS methodology detailed in Chapter 2.

5.3.5. Method validation through spike recovery experiments

After initial optimization experiments, the method employing the developed cleanup cartridges was validated according to EU regulation 644/2017 in both fish and fish oil matrices through spike recovery experiments. According to EU regulation, the stipulated maximum levels for the fish matrix are PCDDFs: 3.5 pg TEQ/g wet weight, PCDD/Fs+dl PCBs: 6.5 pg TEQ/g wet weight, NDL PCBs: 75 ng/g weight, and for the fish oil matrix, PCDDFs: 5 pg TEQ/g, PCDD/Fs+dl PCBs: 20 pg TEQ/g, NDL PCBs: 175 ng/g, weights are relative to a feed with 12% moisture content. The samples were spiked with respect to these levels and spike recovery experiments were conducted. The results of the spike recovery experiments were critically assessed for compliance with regulation. The critical performance indicators such as method accuracy, relative ion ratio tolerance, noise-free chromatograms, and recovery of internal standards were monitored according to the equations given in section 2.3.5 of Chapter 2

5.4. Results and discussions

5.4.1. Optimization of clean-up material synthesis and quality control

Impregnating silica with H_2SO_4 to achieve both 22% and 44% concentrations presents a challenge due to the deliquescent nature of the acid. The conventional method of dispersion in water followed by distillation is unsuitable because once the material comes into contact with

water, removing it becomes exceedingly difficult. To overcome this issue, a specialized laboratory setup has been devised. This setup allows for the direct addition of sulfuric acid into silica under an inert atmosphere, as depicted in **Fig. 5.1**. The inert atmosphere is maintained by continuously purging the system with N_2 gas. Before introducing the silica, the flask is purged with N_2 gas for 10-15 minutes to eliminate any moisture or residual air. Activated silica is then added, followed by the calculated amount of sulfuric acid step by step. Throughout the addition process, the N_2 pressure is kept at a minimum. After each addition of H_2SO_4, the inlet channel is closed, the nitrogen pressure is increased, and the flask is thoroughly shaken to break up any lumps formed. Once all the sulfuric acid has been added, the flask is shaken vigorously for 5-10 minutes to ensure complete homogenization. Finally, the material is transferred into a glass container, ensuring it remains isolated from atmospheric air or moisture.

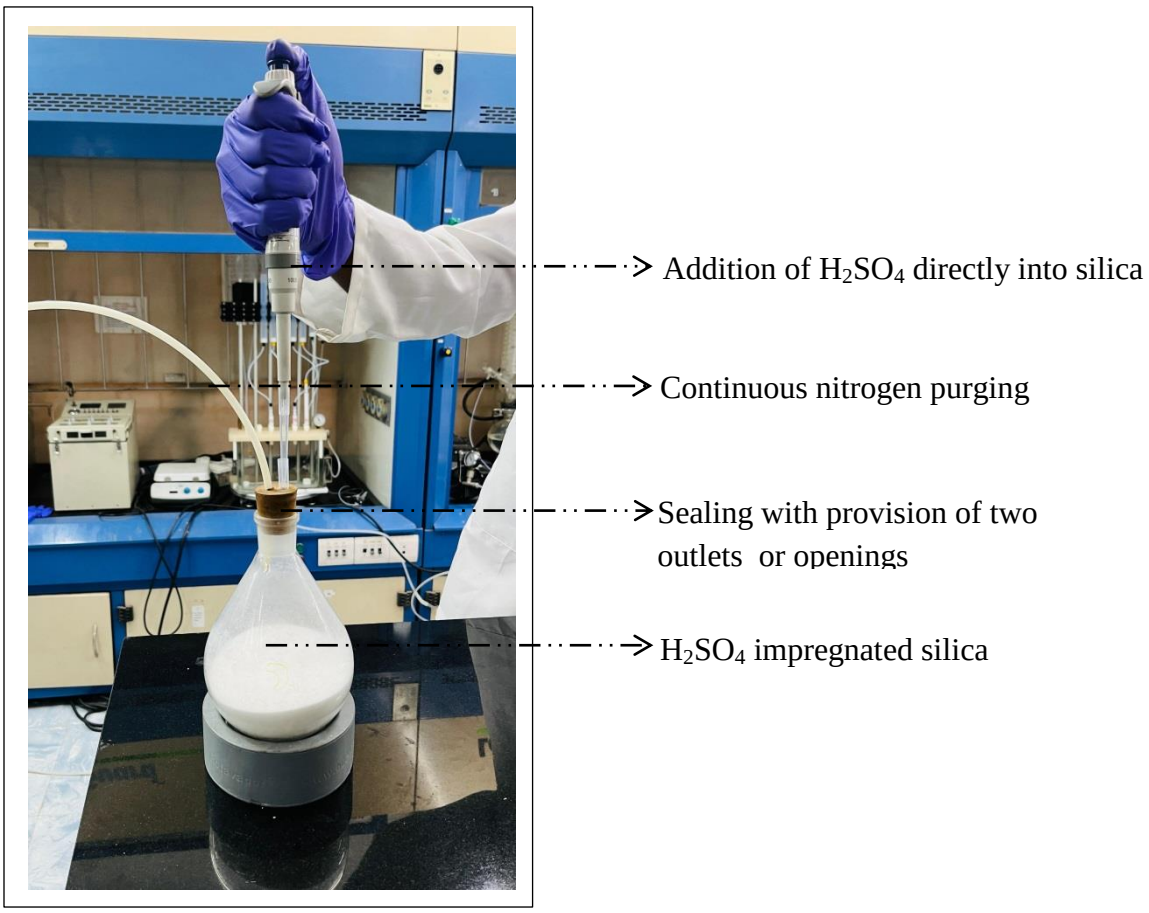


Fig. 5.1. Preparation of sulphuric acid impregnated silica

Synthesized various impregnated silica were characterized using FT-IR technique, the obtained FT IR spectrum was shown in **Fig 5.2**. Broad peaks in the 1000-1200 cm^{-1} range represents Si-O-Si anti symmetric stretching, and peaks near to 800 cm^{-1} represents Si-O-Si symmetric stretching. Sulphuric acid impregnation to silica can be confirmed by stretching modes at 1120–1230 cm^{-1} range, corresponding to O= S =O asymmetric and symmetric stretching modes. FT-IR spectrum of 22 and 44 % H_2SO_4 impregnated silica also shown broad OH stretching absorption around 2800 and 3700 cm^{-1}**.** Since $AgNO_3$ and KOH impregnation is in low percentage, 10% and 2% respectively, characteristic FT-IR peaks are not observed. The synthesized materials were weighed and compared with the theoretical value and ensued moisture adsorption is negligible.

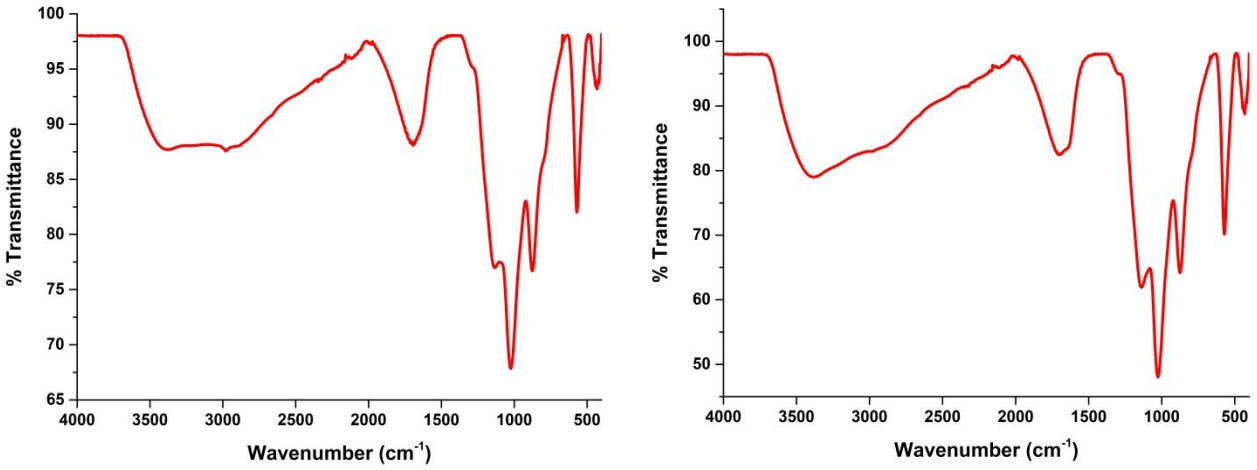


Fig. 5.2 FT IR Spectra a) 22% H_2SO_4 coated silica b) 44% H_2SO_4 coated silica

The alumina utilized in dioxin analysis is basic alumina with activity grade I, determined by the Brockmann and Schodder test. The grading system correlates with moisture content, as outlined in **Table 5.1**. Procured basic alumina with a pH greater than 7 underwent heating at 800°C overnight to eliminate moisture. Verification of the activity grade can be achieved through the Brockmann and Schodder test. In assessing activity, a mixture of two dyes—azo benzene and p-methoxy azo benzene—each weighing 20 mg, was prepared in a 50 ml solvent solution of benzene to petroleum ether in a ratio of 1:4. A glass column with a diameter of 15 mm was packed with alumina to a height of 50 mm. Subsequently, 10 ml of the dye mixture was

introduced into the column, followed by elution with 20 ml of the eluent. Absence of either dye in the eluent designates an activity grade of I. Analogously, other dye mixtures (as detailed in Table 5.1) can be employed to determine the activity of alumina of other grades.

Table 5.1

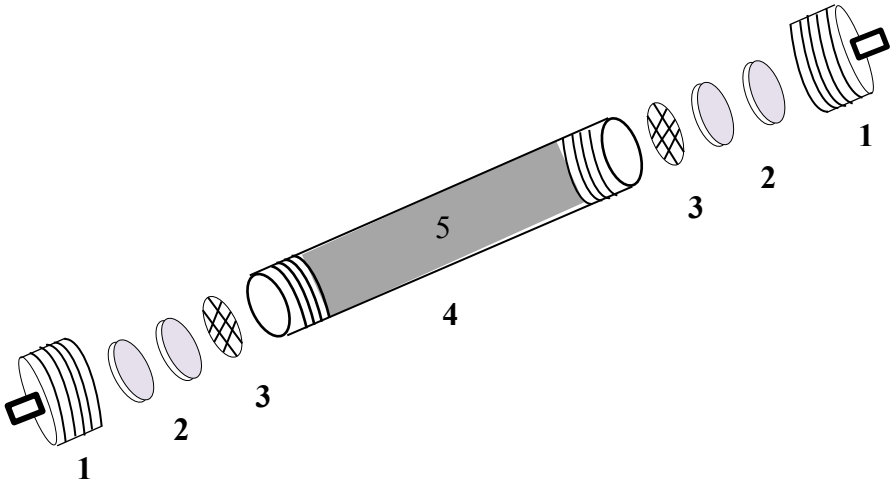
Brockmann and Schodder activity chart

Brockmann activity grade	**I**	**II**	**III**	**IV**	**V**
Moisture content	0%	3%	6%	10%	15%
Dyes need to use assign activity	Azobenzene (AB) and p-methoxyazobenzene (MAB)	p-methoxyazobenzene (MAB) and Sudan yellow (SG)	Sudan yellow (SG) and Sudan red (SR)	Sudan red (SR) and p-aminoazobenzene (AAB)	p-aminoazobenzene (AAB) and p-hydroxyazobenzene (HAB)

5.4.2. Fabrication of clean-up cartridges

The current study utilizes Polyetrafluoroethylene (PTFE) tubing for packing clean-up materials, replacing conventional glass tubes. PTFE tubing offers several benefits, including low weight, breakage-free usability, resistance to organic solvents, and effective sealing capabilities. **Fig. 5.2** presents the schematic diagram of the clean-up cartridge, detailing its components. The tubing is securely connected with frit and glass fiber filter paper at both ends. The frit ensures even dispersion of the solvent, while the glass fiber filter paper prevents sorbent material from escaping during various solvent elution stages. To prevent solvent leakage under high-pressure conditions, both ends of the tubes are securely sealed using end screw caps. A column sealing tool (illustrated in **Fig. 5.4**) was specially designed and fabricated to ensure the secure sealing of the cartridges. In the fabrication process of the multilayer silica, the optimized weight of sorbent layers was packed in the following order: 10% $AgNO_3$ silica, 22% H_2SO_4 silica, 44% H_2SO_4 silica, 2% KOH silica, and Na_2SO_4 layer. Each sorbent layer in the multilayer silica column is separated using silica to prevent intermixing of layers, as depicted in **Fig. 5.3**. For the activated carbon cartridge fabrication, activated carbon mixing with inert diatomaceous earth, increase the

cartridge size and enhancing the interaction rate with planar analytes, resulting in efficient adsorption. Great care was taken during the fabrication of the alumina column. Once activated, alumina is packed into the cartridge in a hot condition; failure to do so may lead to a loss of its activity, affecting its performance in the adsorption of polar analytes. Cartridges were meticulously packed to prevent elution failure due to pressure buildup and to ensure the promotion of an evenly distributed sorbent layer. This was achieved by taping the cartridge during the sorbent packing process, maintaining a consistent and unhindered arrangement. A series of experiments were then conducted to fine-tune each cartridge separately and connected in series as depicted in **Fig. 5.5 (a)**. Optimization experiments aimed to achieve optimal internal standard recovery, prevent pressure failures, and avoid leakage. The optimized dimensions of each cartridge and the filling rate of the sorbent materials are provided in **Table 5.2. Fig. 5.4** further demonstrates the sealing process achieved by sealing tool and fabricated cleanup cartridges.



1. **End screw lock**
2. **Glass fiber filter paper set**
3. **Frit**
4. **PTFE tubing**
5. **Packing material**

Fig.5.2. Schematic representation of fabricate cartridge

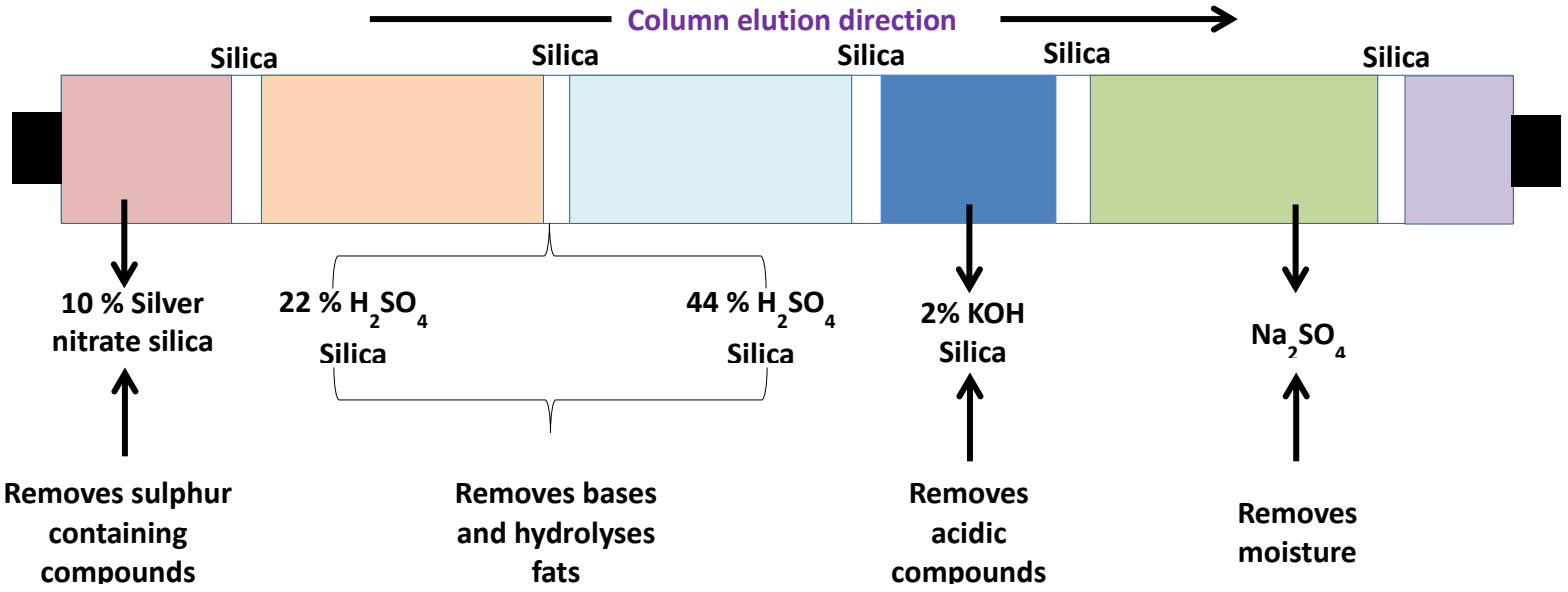


Fig.5.3. Multilayer silica column components and corresponding functions

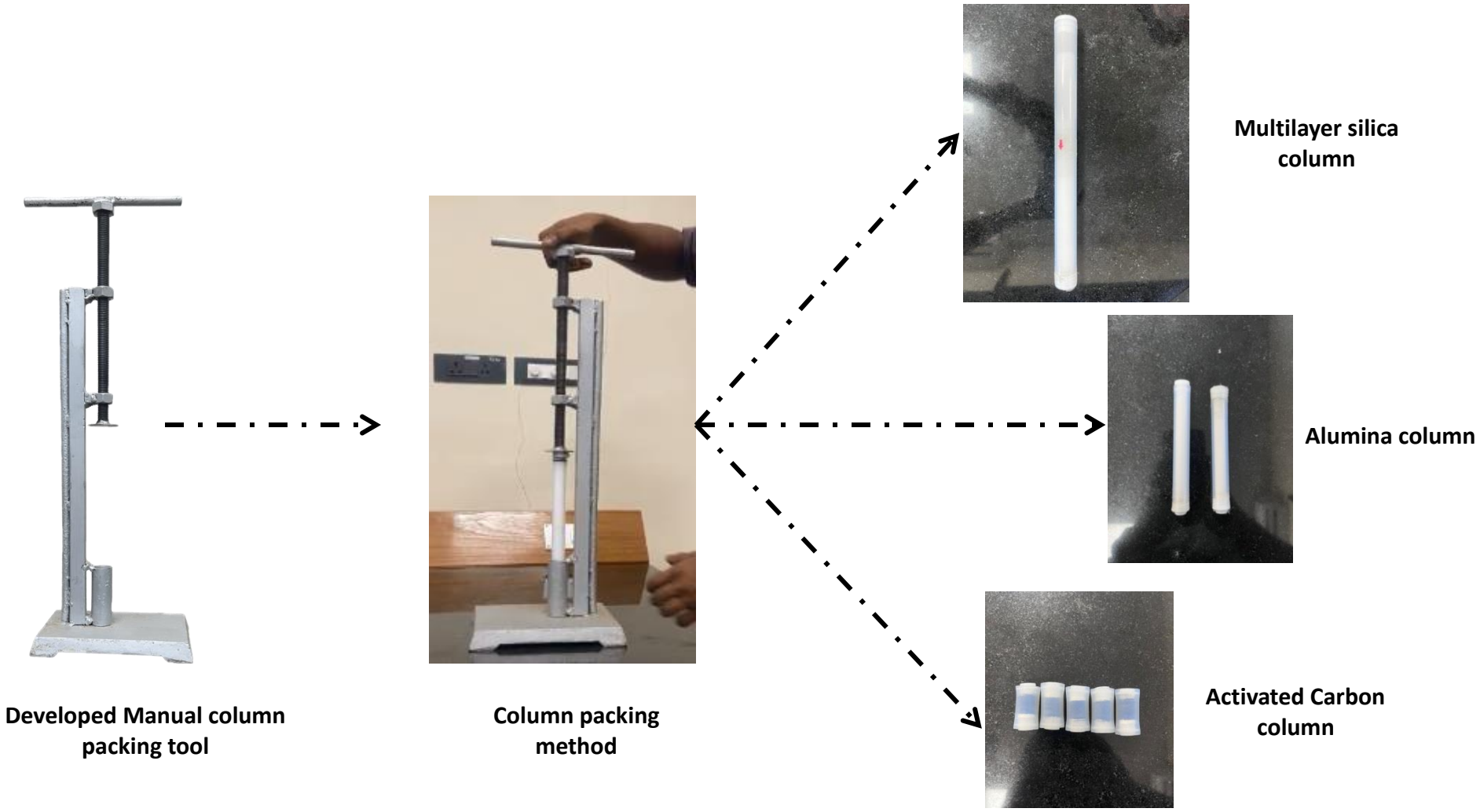


Fig.5.4. Column Fabrication and developed cartridges

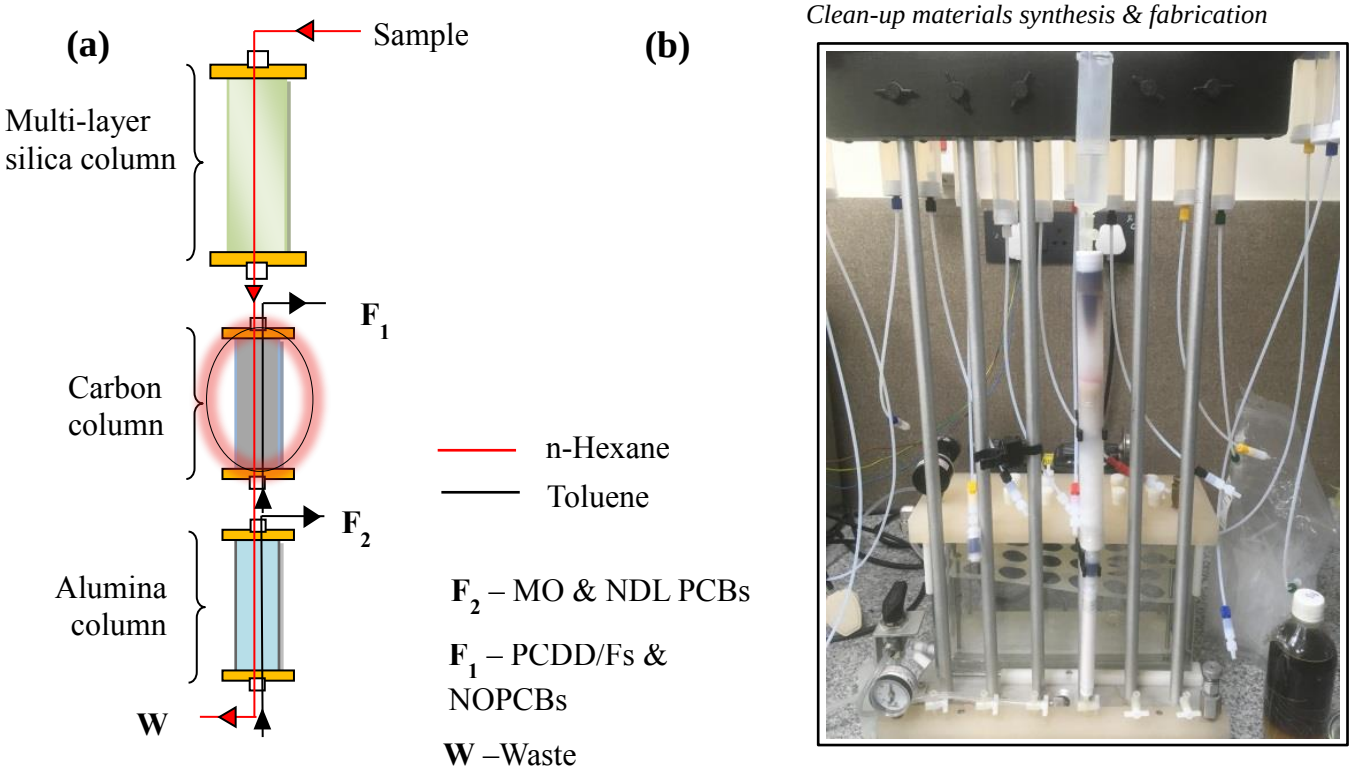


Fig.5.5. (a) Column assembly & fractionation **(b)** Sample analysis with prepared cartridges

Table 5.2

Optimized cartridge dimension, filling rate and solvent volumes

SI No	Cartridge type	PTFE tube dimension	Filling rate
1	Multi-layer silica column	ID= 1.8 cm OD= 2.2 cm Length = 30 cm	10% $AgNO_3$ silica = 5g 22% H_2SO_4 silica = 20g 44%H_2SO_4 silica = 14g 2% KOH silica = 9g, Na_2SO_4 = 3g Silica layer between layers = 0.5 g
2	Activated carbon column	ID= 1 cm OD= 1.4 cm Length = 4 cm	0.5 g AC in 3 g diatomaceous earth
3	Alumina column	ID= 1 cm OD= 1.4 cm Length = 12 cm	4.5 g
Optimized solvent volumes (To process 2.5 g to 3.5 g fat)			
Column assembly conditioning (hexane)= 60 ml Sample introduction (hexane) = 30 ml Forward elution (hexane) = 180 ml Reverse elution of alumina & carbon column =40ml (toluene)			

5.4.3. Initial fractionation trials with activated carbon and alumina cartridges

Fig. 5.6 depicts the schematic overview of the initial fractionation trials conducted using a fabricated cartridge. The fractions obtained were concentrated and subsequently injected into the GC-MS/MS for quantification. Notably, in both FEF1 and FEF2, PCDD/Fs were absent, while in the lower carbon load cartridge FEF2 contained NO-PCBs (Fig. 5.7). This absence may be attributed to their low affinity, likely stemming from reduced planarity. While increasing the carbon load changes this trend by observing a high recovery rate of NO-PCBs in REF. Chromatograms obtained from the high AC load are presented in Fig. 5.9-5.10. Forward elution displays no peaks, whereas reverse elution with toluene exhibits clear recovery of analytes. It's worth noting that achieving complete trapping of NO-PCBs with a carbon column proved challenging. However, optimizing elution volume and AC carbon load could lead to optimal NO-PCB recovery. Experiments utilizing the alumina column, as depicted in **Fig. 5.6**, did not demonstrate discrepancy as that of AC cartridge. The **Fig. 5.8** and chromatograms obtained (**Fig 5.11-5.12**) reveal that forward elution is devoid of MO and NDL PCBs, while optimal recoveries of analytes were observed during reverse elution..

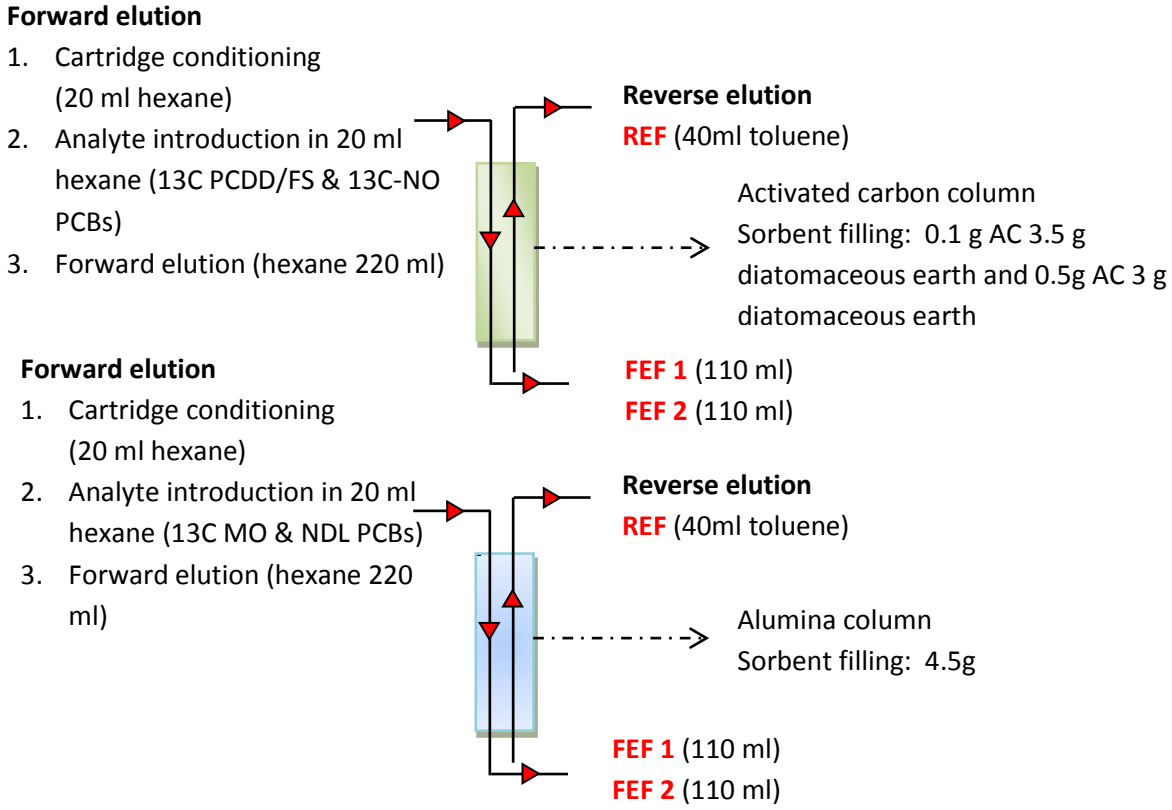


Fig 5.6 Schematic overview of initial optimization experiments with fabricated activated carbon and alumina cartridges (FEF-Forward Elution Fraction, REF- Reverse Elution Fraction)

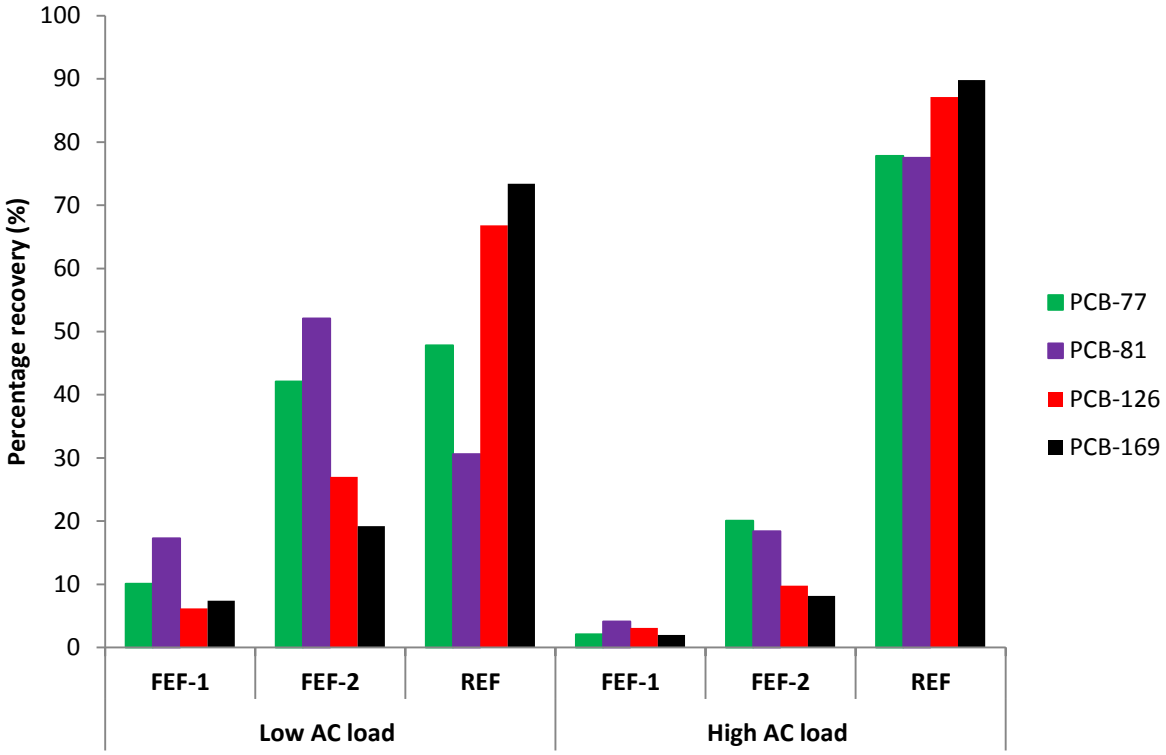


Fig 5.7 Optimization experiments with activated carbon cartridge with variable AC load

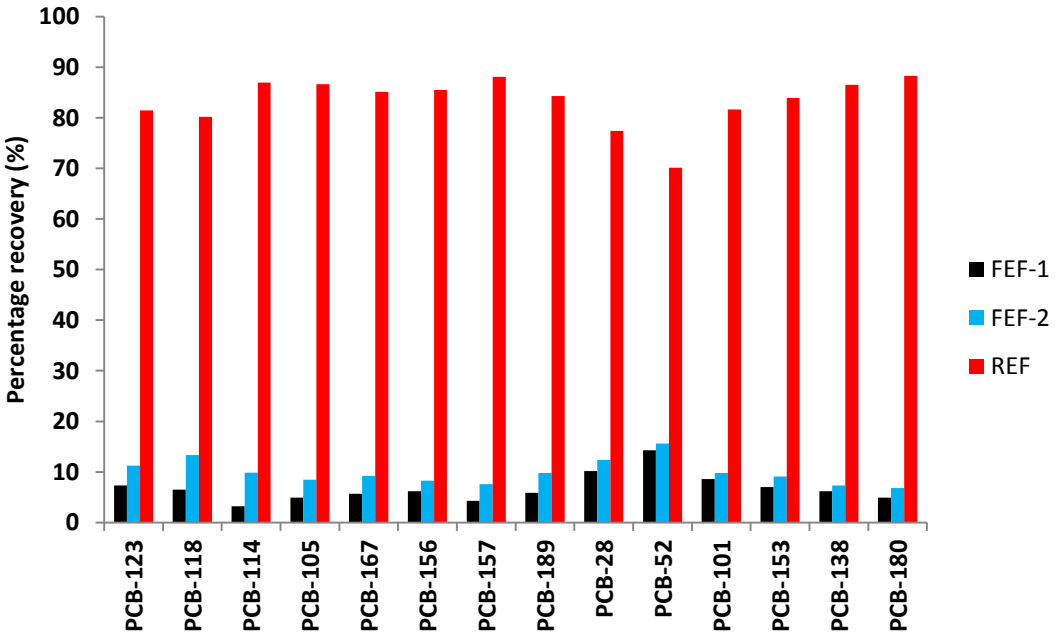


Fig 5.8 Optimization experiments with alumina column

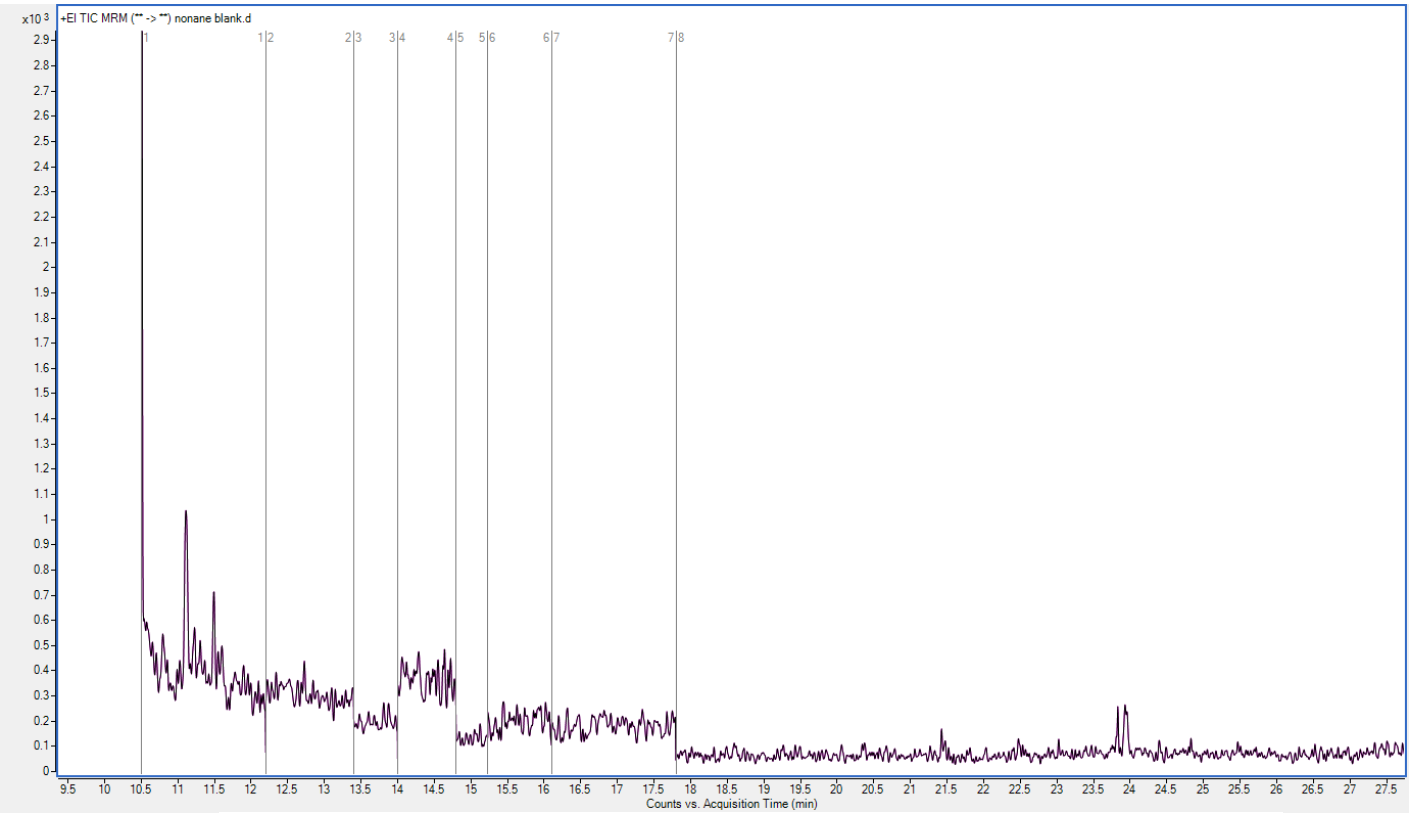


Fig 5.9 Obtained chromatogram of forward elution fraction (High AC load)

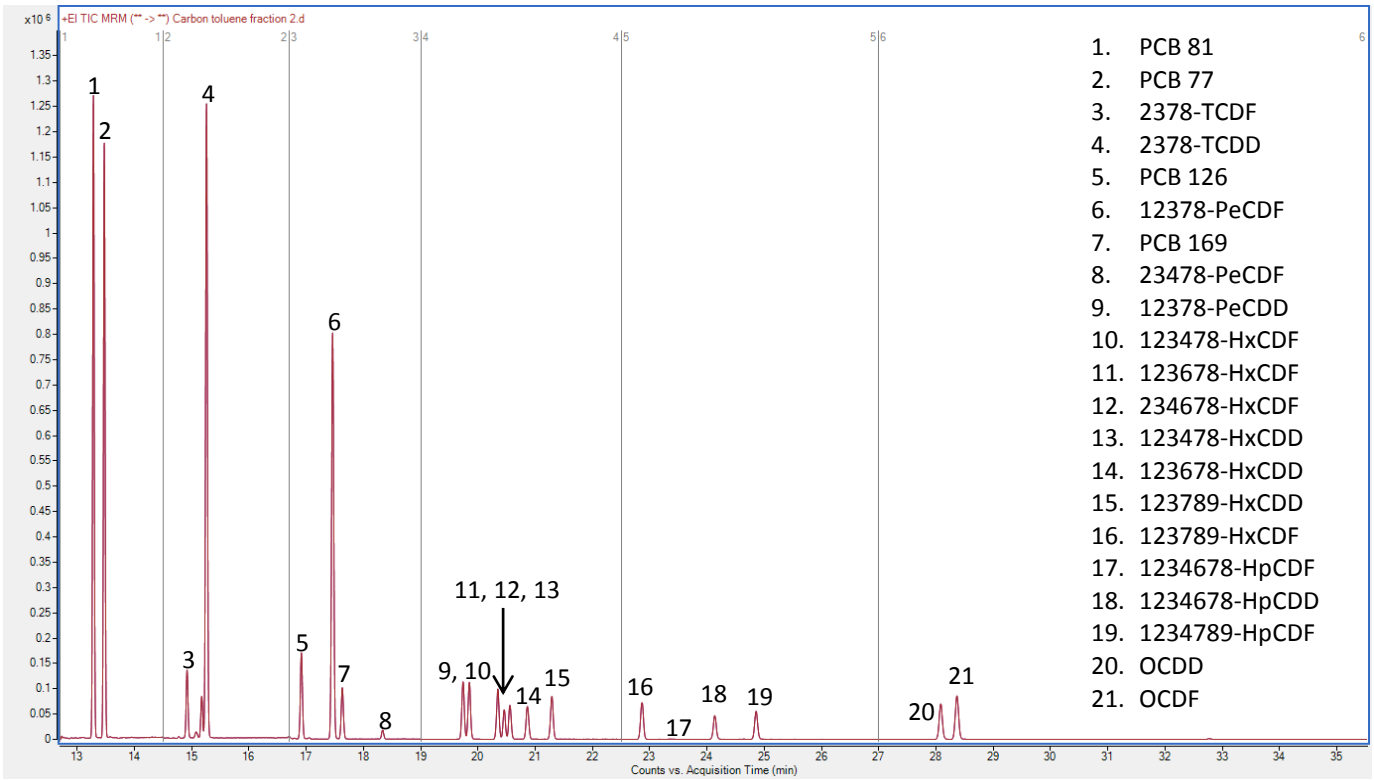


Fig 5.10 Obtained chromatogram of reverse elution fraction (High AC load)

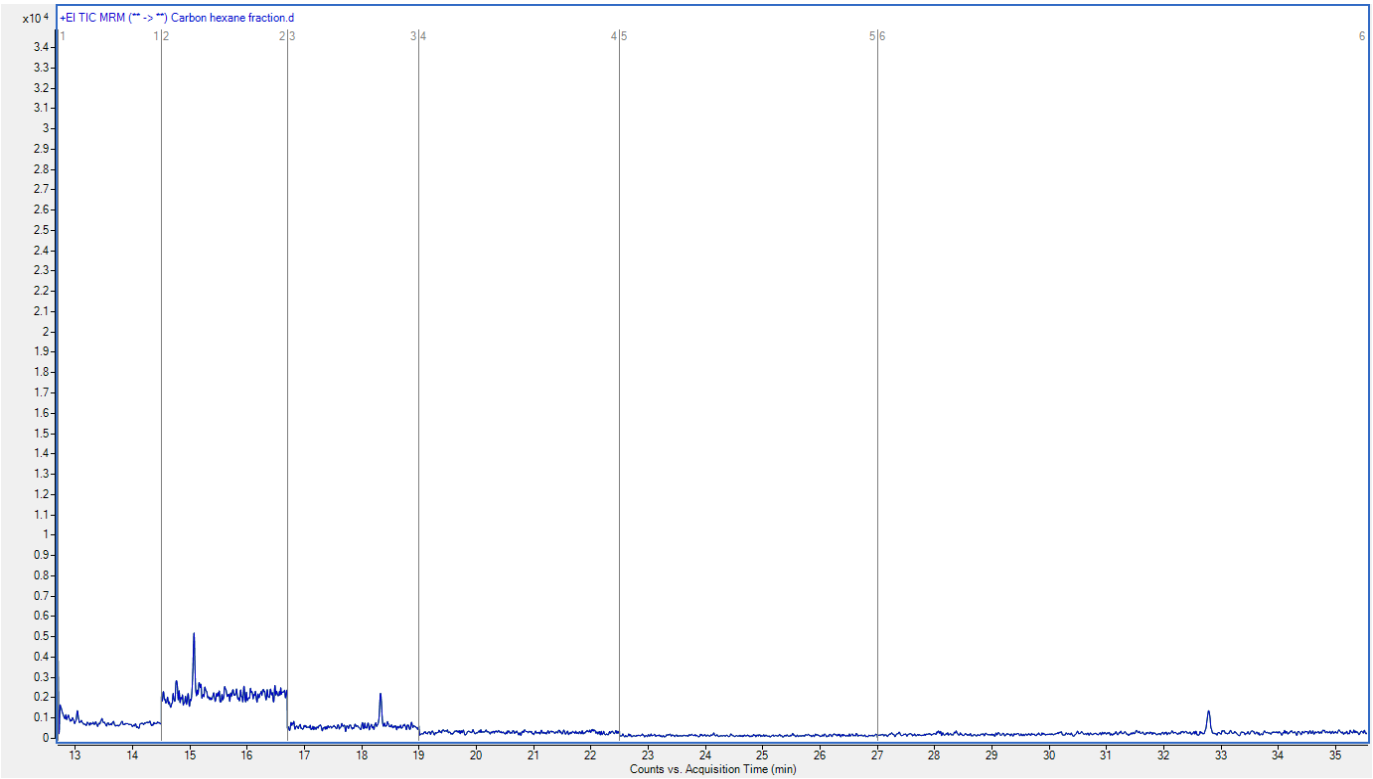


Fig 5.11. Obtained chromatogram of forward elution fraction (alumina

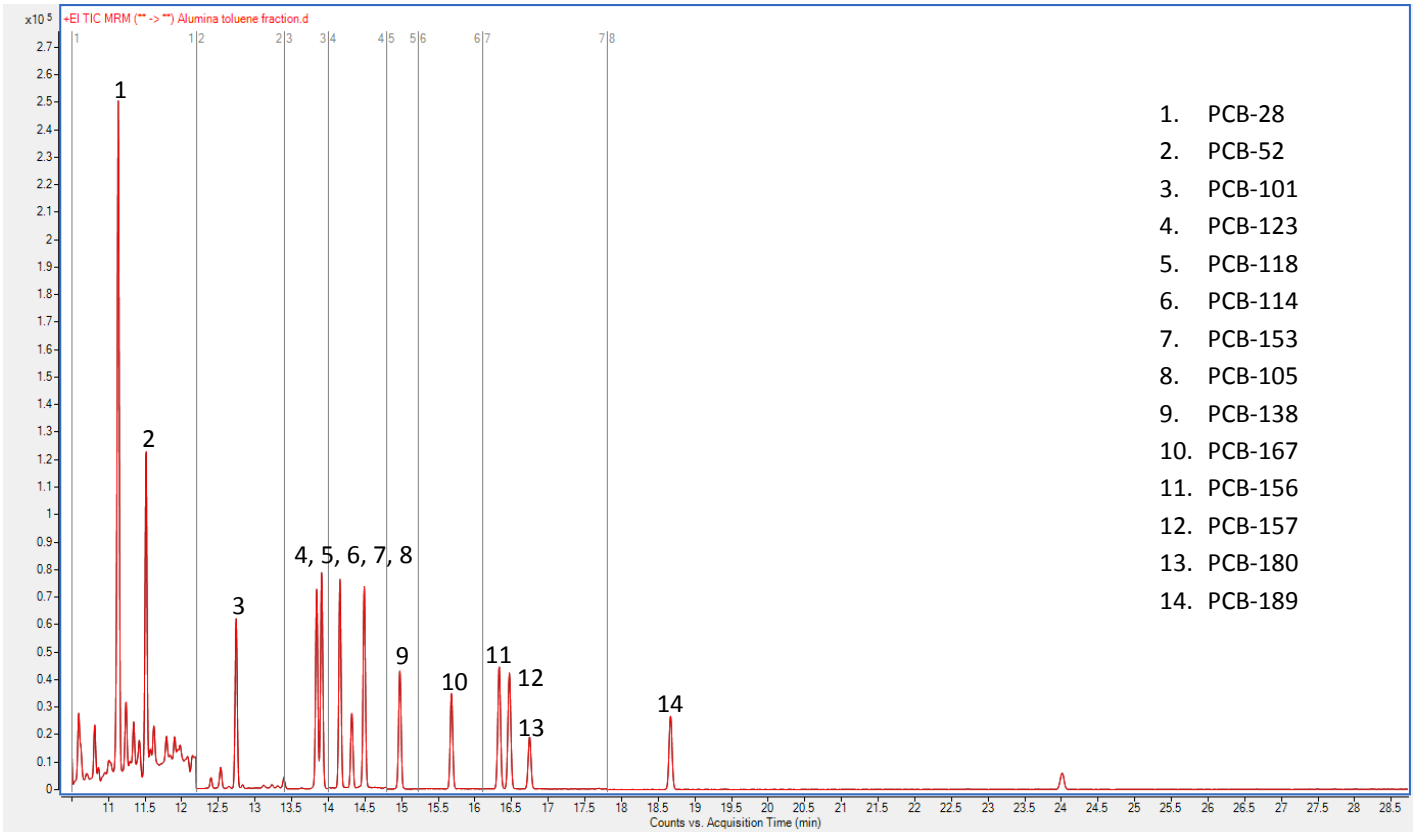


Fig5.12. Obtained chromatogram of reverse elution fraction (alumina column)

5.4.4. Spike recovery experiments and method validation results

Table 5.3. compiles the method validation data derived from spike recovery experiments conducted at maximum levels in fish and fish oil matrices. Three crucial parameters influenced by sample cleanup were assessed: method accuracy (in terms of bias %), relative ion intensity to standard recovery, internal tolerance, and internal standard recovery. The method accuracy, expressed as bias %, for fish matrix, was determined to be -8.9% (Σ PCDD/Fs TEQ), -2.4% (Σ PCDD/Fs + dl-PCBs TEQ), and -1.6% (Σ ndl-PCBs). Similarly, for fish oil matrix, the bias % values were -6.2% (Σ PCDD/Fs TEQ), -2.2% (Σ PCDD/Fs + dl-PCBs TEQ), and -2.8% (Σ ndl-PCBs). These results were well below the regulatory limit of ± 20%. The individual congener bias ranged from -12.3% to 15.7% for fish and -11.0% to 7.5% for fish oil, aligning with EU regulations and demonstrating the efficacy of the developed method. Relative ion intensities of each congener were found to be less than < 15%, meeting the regulatory stipulated limit. This indicates the performance of the cleanup methodology in effectively removing the effects of matrix or co-extracts. The parameter internal standard recovery %represents the loss of analyte during the overall analytical process. The obtained ISTD recoveries fell within the bracket of 60-120%, demonstrating the efficiency of the methodology. Fig. 13 and 14 depict representative, noise-free total ion current (TIC) chromatograms obtained in spike recovery experiments conducted in fish oil matrix, further demonstrating the effective removal of co-extracts. In essence, the monitored performance figures underscore the efficacy of the developed cartridges in removing co-extracts and properly fractionating analytes based on planarity.

.5.4.5. Comparison of performance and costing with respect to commercial cartridges

The performance figures observed in spike recovery experiments and routine analyses conducted in the laboratory indicate that the developed cartridges perform comparably to commercially available ones. **Table 5.4** presents the cost of commercially available cartridges or cleanup materials. Typically, a set of three cartridges (multilayer silica, activated carbon, and alumina) costs approximately ₹10,000-12,500. In contrast, the cost of the developed cartridge set falls within the range of ₹750-1,000, which could be further reduced if the cartridge is prepared commercially. Therefore, indigenous preparation of cleanup materials can significantly decrease the analysis cost.

Table 5.3

Method validation results through spike recovery experiments at maximum level in fish and fish oil matrices employing developed clean-up methodology

SI No	Compound name	WHO TEF_{2005} Values	Ion ratio (Quantifier to qualifier)			Relative ion intensity tolerance (%)		ISTD recovery (%)		Native analyte spiked		Obtained experimental result		Bias (%)	
			Calibration average	Fish	Fish oil	Fish	Fish oil	Fish	Fish oil	Fish	Fish oil	Fish	Fish oil	Fish	Fish oil
1	2378-TCDF	1	62.2	64.2	67.8	3.14	8.93	86.4	108.7	0.56	0.80	0.49	0.714	-12.5	-10.7
2	2378-TCDD	0.1	61.8	63.9	67.4	3.34	9.00	74.3	101.5	0.56	0.80	0.51	0.732	-8.9	-8.5
3	12378-PeCDF	1	96.4	91.4	93.8	-5.19	-2.70	80.2	75.3	1.40	2.00	1.23	1.85	-12.1	-7.5
4	23478-PeCDF	0.03	78.8	83.5	79.3	5.86	0.55	73.7	99.4	1.40	2.00	1.32	1.94	-5.7	-3.0
5	12378-PeCDD	0.3	94.2	90.1	93.9	-4.27	-0.28	80.2	85.2	1.40	2.00	1.25	1.88	-10.7	-6.0
6	123478-HxCDF	0.1	82.5	87.3	89.2	5.70	8.06	91.9	78.5	1.40	2.00	1.25	1.79	-10.7	-10.5
7	123678-HxCDF	0.1	81.3	85.3	86.2	4.99	6.11	76.3	92.4	1.40	2.00	1.32	2.14	-5.7	7.1
8	234678-HxCDF	0.1	72.5	77.2	76.2	6.58	5.18	83.9	71.4	1.40	2.00	1.54	2.15	10.1	7.6
9	123478-HxCDD	0.1	75.2	79.3	76.9	5.33	2.20	89.9	81.4	1.40	2.00	1.36	2.08	-2.8	4.0
10	123678-HxCDD	0.1	62.1	68.5	68.2	10.25	9.81	96.3	108.2	1.40	2.00	1.48	1.78	5.8	-11.0
11	123789-HxCDD	0.1	64.1	66.3	66.2	3.29	3.26	99.3	99.8	1.40	2.00	1.39	1.79	-0.7	-10.5
12	123789-HxCDF	0.1	63.4	69.5	65.3	9.60	3.02	95.3	99.9	1.40	2.00	1.29	1.95	-7.8	-2.5
13	1234678-HpCDF	0.01	65.1	67.5	69.2	3.68	6.34	95.3	77.6	1.40	2.00	1.43	1.92	2.2	-4.0
14	1234678-HpCDD	0.01	66.2	69.5	69.3	4.89	4.54	93.8	105.6	1.40	2.00	1.21	1.79	-13.5	-10.5
15	1234789-HpCDF	0.01	64.5	70.1	59.2	8.76	-8.17	100.0	86.1	1.40	2.00	1.28	2.14	-8.5	7.1
16	OCDD	0.0003	61.9	65.9	56.2	6.47	-9.12	72.0	76.2	2.80	4.00	2.64	3.98	-5.7	-0.5
17	OCDF	0.0003	76.5	79.5	69.2	3.95	-9.45	98.6	76.5	2.80	4.00	2.78	3.78	-0.7	-5.5
								∑PCDD/Fs TEQ		**3.50**	**5.0**	**3.2**	**4.7**	**-8.9**	**-6.2**
18	PCB 81	0.0001	84.9	89.5	80.2	5.31	-5.53	79.2	109.7	22.96	114.82	20.28	112.34	-11.7	-2.2
19	PCB 77	0.0003	78.9	83.9	76.2	6.34	-3.44	87.6	76.8	22.96	114.82	21.89	110.45	-4.7	-3.8
20	PCB 126	0.1	95.7	99.5	98.3	3.94	2.74	89.6	103.3	22.96	114.82	23.92	113.23	4.2	-1.4
21	PCB 169	0.03	94.5	89.5	94.2	-5.34	-0.30	72.0	77.3	22.96	114.82	24.89	115.63	8.4	0.7

22	PCB-123	0.00003	98.6	96.2	98.5	-2.42	-0.17	109.1	103.8	22.96	114.82	23.59	110.34	2.7	-3.9
23	PCB-118	0.00003	96.2	98.5	99.2	2.36	3.15	109.8	86.5	22.96	114.82	22.99	117.23	0.1	2.1
24	PCB-114	0.00003	95.7	90.1	97.5	-5.87	1.78	99.9	75.0	22.96	114.82	26.58	116.34	15.7	1.3
25	PCB-105	0.00003	95.3	99.2	97.2	4.16	2.06	103.5	93.9	22.96	114.82	24.79	118.56	8.0	3.3
26	PCB-167	0.00003	78.5	83.6	83.3	6.47	6.09	87.8	85.4	22.96	114.82	23.69	118.9	3.2	3.6
27	PCB-156	0.00003	77.7	79.5	79.4	2.29	2.14	77.2	86.3	22.96	114.82	20.14	119.34	-12.3	3.9
28	PCB-157	0.00003	79.9	83.5	72.2	4.52	-9.66	88.9	85.7	22.96	114.82	22.84	113.56	-0.5	-1.1
29	PCB-189	0.00003	83.2	79.5	84.2	-4.47	1.25	73.2	74.8	22.96	114.82	21.65	117.29	-5.7	2.2
								∑dl-PCBs TEQ		**3.00**	**15.0**	**3.2**	**14.9**	**5.1**	**-0.9**
								∑PCDD/Fs + dl-PCBs TEQ		**6.50**	**20.0**	**6.3**	**19.6**	**-2.4**	**-2.2**
30	PCB-28	NA	64.5	60.2	69.5	-6.60	7.75	76.6	74.5	12500.0	29166.7	12000.9	28669.2	-4.0	-1.7
31	PCB-52		63.4	59.3	69.5	-6.51	9.71	79.6	89.4	12500.0	29166.7	11899.2	27956.8	-4.8	-4.1
32	PCB-101		94.8	89.5	90.5	-5.65	-4.52	88.2	94.2	12500.0	29166.7	12712.9	29012.3	1.7	-0.5
33	PCB-153		75.7	79.5	79.2	5.03	4.70	70.0	70.9	12500.0	29166.7	12435.2	28445.3	-0.5	-2.5
34	PCB-138		79.9	86.5	73.3	8.23	-8.31	76.0	95.7	12500.0	29166.7	12835.2	27845.9	2.7	-4.5
35	PCB-180		65.2	72.6	60.5	11.31	-7.29	81.9	92.1	12500.0	29166.7	11923.6	28212.6	-4.6	-3.3
								∑ndl-PCBs		**75000.0**	**175000.0**	**73807.0**	**170142.0**	**-1.6**	**-2.8**

Concentration Units : Fish (pg/g wet wight), Fish oil (pg/g, relative to a feed with 12% moisture content)

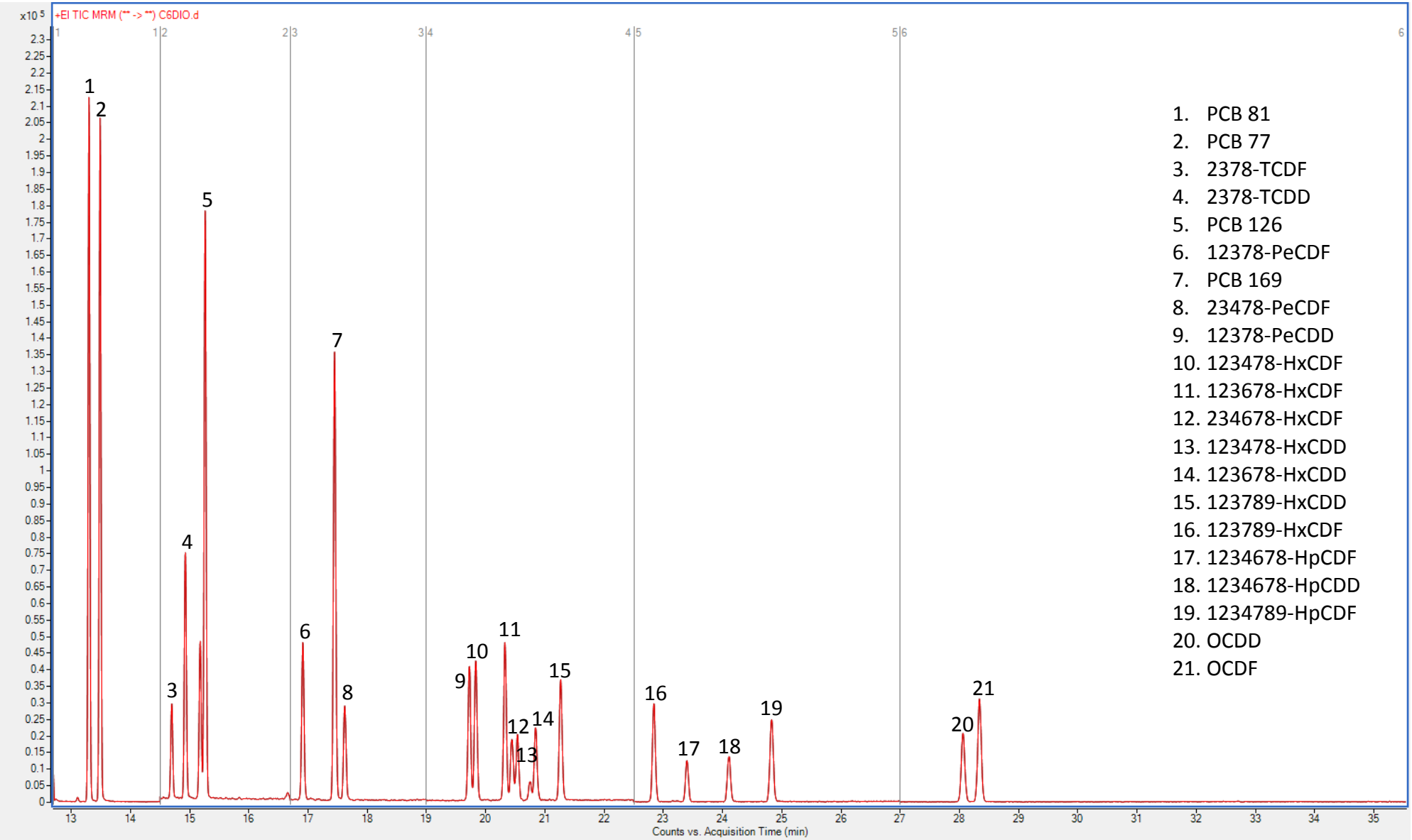


Fig 5.13. Noise free TIC chromatogram obtained for fraction 1 (Dioxins and NO-PCBs): ML spike recovery experiment, fish oil

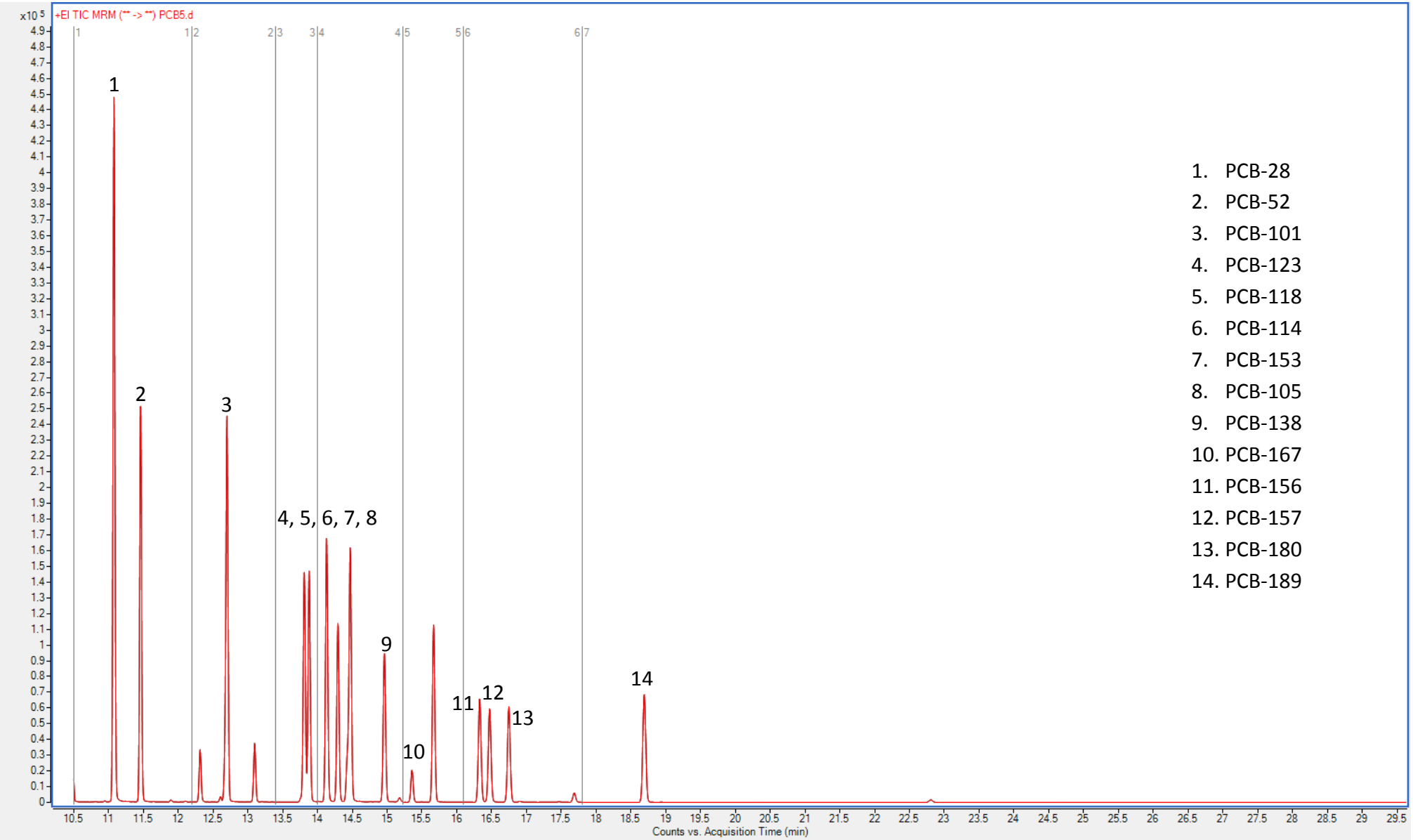


Fig 5.14. Noise free TIC chromatogram obtained for fraction 2 (MO and NDL-PCBs): ML spike recovery experiment, fish oil

Table 5.4 : Commercially available dioxin sample preparation materials and their cost

SI No	Dioxin/PCB cleanup material	Company name	Market Cost	Refernces
	Commercially available materials for dioxin sample preparation			
1	22% Sulfuric Acid-impregnated Silica Gel	FUJIFILM Wako Chemicals U.S.A. Corporation	201 USD (₹ 16691) per 100g	
		Merck (Supelco)	₹29,903.40 per 100 g	
2	44 % Sulfuric Acid-impregnated Silica Gel	FUJIFILM Wako Chemicals U.S.A. Corporation	213 USD (₹17687) per 100 g	
		Merck (Supelco)	₹29,903.40 per 100 g	Dioxin & PCB Analysis, Fujifilm
3	KOH impregnated Silica Gel	FUJIFILM Wako Chemicals U.S.A. Corporation	₹11802.62) per 100 g	
		Merck (Supelco)	₹ 30180.90 per 100 g	Dioxin Sample Preparation Kit, Supelco
4	$AgNO_3$ impregnated Silica Gel	FUJIFILM Wako Chemicals U.S.A. Corporation	₹11802.62 per 100 g	
		Merck (Supelco)	₹30,180.90 per 100 g	
5	Alumina B – Super I	Sorbtech	220USD (₹18260) per 500 g	
		MP chemicals	₹ 2311 per 100 g	
	Commercially available pre packed cartridges			
6	Multi-Layer Silica Gel Dioxin Column	Merck (Supelco)	₹29,822.88 per 5 columns	Dioxin Sample Preparation Kit, Supelco
7	Dual-Layer Carbon Reversible Tube	Merck (Supelco)	₹32,864.70 for 10 columns	
8	Present study (Pack of three cartrdiges	-	₹750-1000	

5.5. Conclusions

In conclusion, this chapter introduces a cost-effective cleanup protocol with performance comparable to commercially available clean-up cartridges. Synthetic strategies were developed to address the challenges deteriorating the quality of the material. Several optimization

experiments were carried out to ensure efficient fabrication of clean-up materials in PTFE tubes. Furthermore, the developed methodology is validated in two critical food and feed matrices, fish and fish oil, respectively, in accordance with EU regulation. The assessed critical parameters such as method accuracy, internal standard recovery, and relative ion intensity values are in compliance with the regulation, demonstrating the efficacy of the developed method. In essence, this study sets a benchmark in achieving low-cost dioxin analysis in critical matrices.

Chapter

Conclusions

The widespread surveillance of dioxins and PCBs, categorized as Persistent Organic Pollutants (POPs), is significantly impeded by the high analysis costs associated with confirmatory analysis. The hindrance arises from the use of sophisticated analytical instrumentation, such as GC- magnetic sector HRMS, and expensive sample cleanup protocols. However, recent advances in mass spectrometry have introduced a cost-effective alternative—GC coupled with triple quadrupole mass spectrometer (GC-MS/MS)—for the analysis of dioxins and PCBs. Regulatory bodies, including the European Union, have acknowledged the efficacy of this analytical technique for confirmatory quantification in various food matrices, provided the methodology adheres to stringent regulatory criteria. Despite these advancements, limited studies have been conducted to establish GC-MS/MS-based confirmatory quantitation of these contaminants in food matrices, and there is a notable absence of research exploring the applicability of this methodology as an analytical tool to assess the current status of dioxin-like POPs hotspots. Furthermore, most dioxin/PCBs analysis laboratories rely on commercially available cleanup modules integrated with commercial chromatographic cartridges. The indigenization of the cleanup step through in-house synthesis and fabrication of cleanup materials has the potential to significantly reduce analysis costs. Although numerous reports affirm the efficiency of activated carbon as an adsorbent for aromatic contaminants, there is a scarcity of studies focusing on the congener-wise adsorption characteristics of dioxins on activated carbon. Additionally, in-depth studies on the effective use of the adsorption property of activated carbon to facilitate the decontamination of this class of pollutants from feed matrices are limited. The thesis addresses these research gaps as follows.

The first chapter covers a detailed introduction and literature review focusing on characteristics of dioxins and PCBs, recent out breaks of these contaminants in food and feed and analytical procedures to quantify these. The first working chapter of the thesis deals with the development and critical validation of a GC-MS/MS-based analytical method for fish and fish oil matrices. The validation criteria, outlined in relevant EU regulations, are broadly categorized into three aspects: basic requirements for confirmatory methods, requirements for GC-based methods, and specific requirements for GC-MS/MS-based methods. The developed method successfully met all the stipulated validation criteria, including calibration efficiency, limit of quantification (LOQ), precision, accuracy, ion ratio, and recovery rates. The calculated method performance

matrices such as bias % and RSD % align with regulatory criteria, affirming the accuracy and precision of the developed method.

Chapter 3 focuses on the field testing of the developed methodology by analyzing fish and sediment samples from the Eloor-Edayar industrial belt, a solitary POPs hotspot in India. The profiles of congeners indicate that dl-POPs were formed through precursor pathways, suggesting the potential release of chlorinated precursor species from the surrounding industrial area as the root cause. Fish samples from hotspots were observed to have 8 times higher levels of PCDD/Fs) and 30 times higher levels of PCBs than the control sites. A strong statistically significant ($p < 0.05$) positive correlation was observed between dl-POPs levels in fish and sediment samples at the study site, and the BSAF for PCDD/Fs and dl-PCBs ranged from 0.019 to 0.092 and 0.004 to 0.671 respectively. The estimated weekly intake from fish consumption in the study region was observed to be 3 to 24 times higher than the maximum levels set by the European food safety authority (2 pgTEQ $kg^{-1}bwweek^{-1}$).

Chapter 4 delves into the versatile applications of activated carbon, specifically derived from coconut shells, unveiling its dual functionality in sample cleanup and the decontamination of dioxins and non-ortho polychlorinated biphenyls (NO-PCBs) from fish oil matrices. The chemical activation process using KOH enhanced the surface characteristics and textural properties of the activated carbon, resulting in a surface area of 390 m^2/g and a total pore volume of 0.219 cm^3/g. Batch adsorption experiments demonstrated the efficacy of the activated carbon, aligning with pseudo second-order kinetics and the Langmuir isotherm model. This activated carbon was seamlessly integrated into PTFE tubing, creating a user-friendly cartridge for sample preparation. Validation of the sample preparation step was achieved through spike recovery experiments. The developed activated carbon showcased remarkable potential in decontaminating dioxins and NO-PCBs in fish oil matrices, with an optimized ratio of AC to fish oil at 0.1:21 achieving 90-98% adsorptions. Crucially, assessments of fish oil quality parameters—fatty acid profile, Free Fatty Acid (FFA % Oleic), Peroxide Value (PV), and Acid Value (AV)—before and after the decontamination process revealed no significant differences. Thus, the adopted decontamination strategy demonstrated effectiveness without compromising the quality of fish oil.

Chapter 5 is dedicated to the indigenization of the sample cleanup protocol, achieved through the synthesis of cleanup materials and their fabrication into ready-to-use sample cartridges. The individual components of the multi-layer silica cartridge, including 22%, 44% -H_2SO_4, 10%-KOH, and 10%-$AgNO_3$ impregnated silica, underwent characterization using the FT-IR technique. Basic alumina (Activity I) was synthesized following the Brockmann activity scale. These synthesized materials were then meticulously packed into PTFE tubings, ensuring the avoidance of elution failure due to pressure build-up and promoting an evenly distributed sorbent layer. The packing process involved careful taping of the cartridges to maintain a consistent and unhindered arrangement. The efficacy of the developed cartridges was assessed through experiments with fish/fish oil matrices and validated in accordance with EU regulations.

In summary, this thesis addresses critical challenges in the surveillance of Persistent Organic Pollutants (POPs), particularly focusing on Dioxins and Poly Chlorinated Biphenyls (PCBs). The research unfolds by introducing an innovative and cost-effective GC-MS/MS-based analytical method for the precise quantification of these contaminants in fish and fish oil matrices. Further, it delves into a comprehensive assessment of a historical POPs hotspot, shedding light on the potential release of chlorinated precursor species in industrial areas. Additionally, the study explores the multifaceted applications of activated carbon derived from coconut shells. This includes its dual functionality in both sample cleanup and the effective decontamination of dioxins and non-ortho polychlorinated biphenyls (NO-PCBs) from fish oil matrices. The chemical activation process using KOH enhances the surface characteristics of the activated carbon, making it a versatile sorbent material with promising applications in environmental cleanup. Furthermore, the thesis contributes to the indigenization of sample cleanup protocols through the synthesis of cleanup materials and their fabrication into user-friendly sample cartridges. This step aims to reduce analysis costs and enhance efficiency in POPs surveillance. In the concluding remarks, the research emphasizes the successful validation of the developed methods, providing valuable insights into environmental contamination pathways and offering practical solutions for efficient sample cleanup.

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ABSTRACT

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Faculty of study: Chemical Sciences **Year of submission: 2024**

CSIR Lab: CSIR NIIST **Name of the supervisor: Dr K. P. Prathish**

Title of the thesis: Development of GC-MS/MS based affordable confirmatory analytical methodology for dioxins and PCBs in fish and fish products and exploration of a sustainable decontamination strategy

Dioxins and PCBs are unintentionally generated Persistent Organic Pollutants regulated globally due to their adverse health effects at ultra-trace levels. Since they are highly lipophilic, ingestion is considered the major exposure route for human beings. Hence, it is imperative to enhance the surveillance of these toxic species in animal originfood and feed matrices. The hurdle in the widespread surveillance is the elevated analysis cost. The higher analysis cost can be attributed to the high cost associated with GC-HR MS-based confirmatory quantification and the high cost of sample cleanup materials. Regulatory bodies like the European Union have accepted cost-effective GC-MS/MS-based methods as confirmatory analytical methods in the realm of dioxin/PCBs analysis. The present study focuses on the development of a cost-effective analytical protocol for dioxin confirmatory quantification. The thesis deals with the following aspects:

i. Development and validation of GC-MS/MS-based confirmatory analytical methodology in accordance with EU regulation 644/2017 for the quantification of dioxins and PCBs in fish and feed-grade fish oil.
ii. Utilization of these developed methodologies to assess the current status of an identified Persistent Organic Pollutants (POPs) hotspot in India
iii. Development of KOH-based activated carbon and detailed study, including their characterization, adsorption, and kinetic behavior in the adsorption of dioxins, and exploring its application potential in decontamination of feed matrices
iv. Development of cost-effective cleanup materials for dioxin sample preparation and fabrication of the same into ready-to-use cleanup cartridges.

In essence, the thesis introduces a cost-effective analytical method for confirmatory analysis of dioxins and PCBs in fish and fish oil matrices along with a sustainable decontamination strategy for these contaminants from fish oil. This methodology can be extend to different matrices, to ensure widespread surveillance, which is quintessential for protecting both human health and the environment.

List of Publications

Publication emanated from the thesis

1. **P S, Kirankumar,** K, Sanath., S V, Ajay., Varghese, Amala., K P, Prathish., 2023. Development of a field-deployable analytical workflow for determining current status and indicative human health risks at a historic dl-POPs hotspot. Environ. Pollut. 122161. https://doi.org/https://doi.org/10.1016/j.envpol.2023.122161
2. **P. S Kirankumar**, V R Vaishna, R G Anukrishna, K. P. Prathish. Coconut shell based low cost activated carbon as an effective sorbent for dioxin sample preparation: Synthesis, Adsorption and Kinetic studies (Manuscript submitted)
3. **P. S Kirankumar**, K. P. Prathish Development of effective analytical protocol and its step by step validation as per EU regulation for trace level quantification of dioxins and PCBs in fish and sediment matrices: A GC MS-MS based approach (Manuscript under preparation)

Other publications

1. Ajay, S. V., **Kirankumar, P. S**.,Sanath, K., Prathish, K. P., & Haridas, A. (2022). An experimental simulation study of conventional waste burning practices in India for the assessment and inventorisation of PCDDF dl-PCB emissions. Journal of environmental management, 303, 114109 https://doi.org/10.1016/j.jenvman.2021.114109

2. Ajay, S. V., **Kirankumar, P. S**.,Varghese, A., & Prathish, K. P. (2022). Assessment of Dioxin-Like POPs Emissions and Human Exposure Risk from Open Burning of Municipal SolidWastes in Streets and Dumpyard Fire Breakouts. Exposure and Health, 1- 16. https://doi.org/10.1007/s12403-021-00450-4

3. Amala Varghese; **Kirankumar P S**; Ajay S V; K.P. Prathish Foraging animal origin food samples as viable passive indicators of dioxin-like POPs contamination in industry sites: Method development, characterization and risk assessment (Manuscript under preparation)

4. Jiffin Sam, **P.S. Kirankumar**, K. Sanath, K.P. Prathish*, Development of saleable chloride free iron oxide from hazardous waste in titanium industries via layered double hydroxide formation Journal of Environmental Management, 290 (2021), 112566. https://doi.org/10.1016/j.jenvman.2021.112566

5. Mohanraju, K., **Kirankumar, P. S**., Cindrella, L., & Kwon, O. J. (2017). Enhanced electrocatalytic activity of Pt decorated spinals (M_3O_4, M= Mn, Fe, Co)/C for oxygen reduction reaction in PEM fuel cell and their evaluation by hydrodynamic techniques. Journal of Electroanalytical Chemistry, (2017), 164-174, 794 https://doi.org/10.1016/j.jelechem.2017.04.011

Conference Proceedings

1. **Kirankumar P S**, K Sanath, S V Ajay, Amala Varghese , K. P. Prathish A critical study to explore affordable tools to assess dioxin-like POPs bioaccumulation & human health risk trends at POPs hotspots in developing countries Given oral presentation in Kerala Science Congress, 10-14 February 2023
2. **Kirankumar P. S.**, S. V. Ajay, Amala Varghese, K. P. Prathish "Correlation of PCDD/Fs and PCBs levels between fish and sediment samples and health risk assessment to consumers: A prima facie study carried out in an industrial hotspot located in south India by validated GC MS-MS analytical protocol" 2022 North American Chemical Residue Workshop July 25-29, 2022, held virtually (Awarded 2022 NACRW Student Scholarship Award)
3. **Kirankumar P.S.**, K. Sanath, S. V. Ajay, Amala Varghese ,Jiffin Sam , K.P. Prathish "First study in India on analytical method development and validation for the quantification of dioxins and PCBs in fish samples using GC-MS/MS & trends in samples collected from polluted sites" 7th Annual Conference of the AOAC INTERNATIONAL, India Section in New Delhi on February 28 and 29, 2020 (Selected for RSC Analyst best poster price)
4. **Kirankumar P. S**, Jiffin Sam, K. P. Prathish "An innovative process for the value addition of iron oxide waste from titanium industry" 28th Swadeshi Science Congress 2018 on 7-9 November, 2018. (Awarded best oral presentation award)
5. **Kirankumar P. S.**, Jiffin Sam, K. P. Prathish "Green materials for the removal of chlorides from industrial wastewater", International conference on Recent Trends in Material Science and Technology (ICMST-2018), Held on October 10 to 13, 2018

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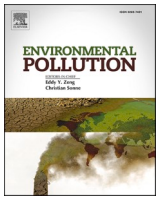

Development of a field-deployable analytical workflow for determining current status and indicative human health risks at a historic dl-POPs hotspot☆

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A B S T R A C T

This paper introduces an integrated workflow that effectively evaluates environmental and health risks of dioxin-like Persistent Organic Pollutants (dl-POPs) at industrial hotspot regions. The developments of validated, cost effective and user-friendly analytical strategies which can be field deployable are quintessential for routine monitoring of dl-POPs, particularly in developing countries. This study addresses the lacunae by enabling an exclusive gas chromatography triple quadrupole mass spectrometer based analytical workflow substituting conventional magnetic sector high resolution mass spectrometer technique and validated the methodology as per the European Union regulation 644/2017. The viable monitoring utility of the methodology for predicting enviro-food-health nexus was field-tested by analyzing fish and sediment samples from the Eloor-Edayar industrial belt, a solitary POPs hotspot in India. The profiles of congeners indicate that dl-POPs were formed through precursor pathways, suggesting the potential release of chlorinated precursor species from surrounding industrial area as the root cause. Fish samples from hotspots were observed to have 8 times higher levels of polychlorinated dibenzo-p-dioxin/furans (PCDD/Fs) and 30 times higher levels of polychlorinated biphenyls (PCBs) than the control sites. A strong statistically significant ($p < 0.05$) positive correlation was observed between dl-POPs levels in fish and sediment samples at the study site and the Biota sediment accumulation factors for PCDD/Fs and dl-PCBs ranged from 0.019 to 0.092 and 0.004 to 0.671 respectively. The estimated weekly intake from fish consumption in the study region was observed to be 3 to 24 times higher than the maximum levels set by the European food safety authority (2 pgTEQ kg^{-1}bwweek^{-1}). Hence, the periodic surveillance of dl-POPs employing user friendly/validated confirmatory tools stands highly imperative to safeguard human health and environment. **Keywords**: Dioxin and PCBs, GC-MS/MS, POPs Hotspot, Biota-sediment accumulation factor, Correlation analysis, Health risk assessment.

1. Introduction

The world we live in today is prevalent with environmental contaminants that can wreak havoc on our food safety and human health, and Polychlorinated dibenzo-dioxin/furans (PCDD/Fs) (17 congeners) and Polychlorinated biphenyls (PCBs) (18 congeners) are among the most dangerous group (Lauby-Secretan et al., 2013). Considering the PBT/LRT (Persistent, Bio accumulative, Toxic and Long-Range Transport) characteristics and adverse health effects imparted at ultra-trace level, these classes of compounds were listed among twelve initial Persistent Organic Pollutants (POPs) under Stockholm convention (Matthies et al., 2016). Due to its extreme toxicity, exposure to dioxin like (dl)-POPs can cause adverse health effects, including chloracne, impaired reproductive function, immune system suppression, and cancer (Bertazzi et al., 2001; Kogevinas, 2001; White and Birnbaum, 2009). Hence regular surveillance of dl-POPs, especially in hotspot/contaminated sites is highly recommended. As part of the world wide POPs monitoring activities, International Pollutant Elimination Network, (IPEN) prepared "World map of POPs waste hotspots" and identified forty POPs hotspots around the world, most of which are located in least developed/developing economies (Watson et al., 2009).

The monitoring of dl-POPs in industrial hotspot regions have received limited attention in previous studies (Fig. (1c).), while the focus was primarily on the dl-POPs hotspots in Vietnam (Van Thuong et al., 2015; Hoang et al., 2014). These studies were primarily driven by the urgent need to address the issue of Agent Orange contamination. Although there are routine surveillance activities for dl-POPs in developed countries, this is not the case in developing/economies in transition. The lack of validated analytical methodology, prohibitively high capital costs (ranging from 500 to 600 K USD), operational and maintenance costs involved in confirmatory analysis of dioxins using the gold standard, High Resolution Gas Chromatograph-High Resolution Mass

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Spectrometer (HRGC-HRMS) pose a great challenge (Palmiotto et al., 2013). With advances in mass spectrometric techniques, GC coupled with triple quadruple mass spectrometer MS (GC-MS/MS) has become an excellent tool for targeted analysis. Studies have shown that GC-MS/MS can also provide results on par with HRMS, which is achieved through its high selectivity and through sample preparation protocols. It possess attractive features such as low installation costs (ranging from 150 to 200 K USD) and reduced operational and maintenance requirements. International organizations like the European Union (EU) and the United States Environmental Protection Agency (USEPA) acknowledge this fact and have laid down performance-based, fit-for-purpose criteria to validate GC-MS/MS for confirmatory analysis of dioxins and PCBs (USEPA, 2022; European Union, 2017). While there are studies focused on validating GC-MS/MS-based methods (Ábalos et al., 2016; Franchina et al., 2019; L'Homme et al., 2015) for specific food and feed matrices, the use of this promising alternative for comprehensive monitoring of dl-POPs pollution is limited. The application of GC-MS/MS in broader surveillance efforts targeting hotspots contaminated with dl-POPs has yet to be adequately explored. Therefore, there is a need to realize the immense potential of GC-MS/MS as a cheap and reliable method to monitor dl-POPs in hotspot regions, thus enabling a more comprehensive assessment of their environmental and health risks.

The present study is focused to establish a cost-effective GC-MS/MS

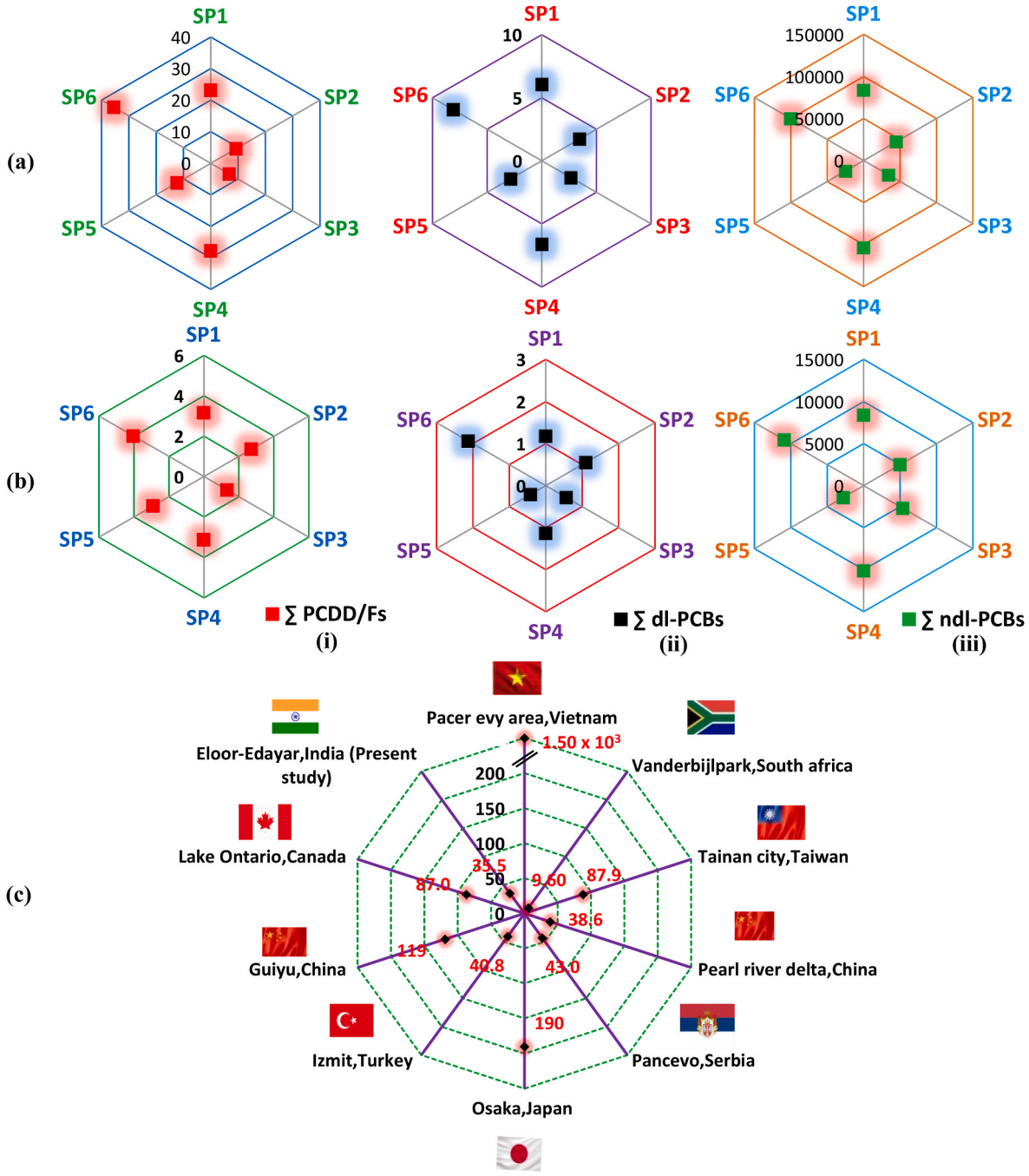

Fig. 1. Present status of hotspot in terms of (i) dioxins (ii) dl-PCBs (iii) ndl-PCB: 1 (a) Sediment samples (d.w basis) 1 (b) fish samples (w.w basis) 1 (c) PCDD/Fs levels observed in sediment samples of various hotspots around the world (pg TEQ/g dw basis). (Bhavsar et al., 2010; Chang et al., 2010; Kaisarevic et al., 2011; Karademir et al., 2013; Kishida et al., 2010; Liu et al., 2022, 2023; Nieuwoudt et al., 2009; Van Thuong et al., 2015).

methodology based analytical workflow for monitoring hotspots with respect to dl-POPs. The field implementation studies were conducted in the Eloor-Edayar region, the only POPs hotspot in India. This region, one among 40 hotspots across the world, is of particular concern due to the presence of dichlorodiphenyltrichloroethane (DDT) and other organochlorine pesticides manufacturing plants, which may result in unintentional generation of dl-POPs (Divya et al., 2021). The DDT manufacturing unit, operated by The Hindustan Insecticides Ltd (HIL), was active until 2018, making it the only global manufacturer of DDT. The unintentional generation of dl-POPs during chlorinated pesticides manufacturing processes is well-known (Liebens and Mohrherr, 2015). However, like many other hotspots worldwide, dl-POPs have been poorly monitored in this region due to the complexity and high cost of confirmatory analysis (Stefanovic et al., 2019).

The methodology developed as part of the current study fills this gap with a critical focus on the development and validation of a GC-MS/MS-based confirmatory tool for fish and sediment matrices according to EU regulations (European Union, 2017). Custom-designed sample preparation steps were employed to optimize the performance of the quantification instrument, GC-MS/MS. The study includes detailed examination of the congener fingerprints, congener group-wise abundance, and correlation patterns of dl-POPs in the fish and sediment samples, together with the Biota Sediment Accumulation Factor (BSAF) values to understand the extent of transfer of contaminants into the food chain. The study also focuses on assessing the health risk for fish consumers in the region by estimating the daily intake and comparing it with the tolerable levels assigned by global stakeholders such as World Health Organization (WHO)/European Food Safety Authority (EFSA). Thus the present study could evolve as a systematic and cost effective analytical workflow for the global monitoring of dl- POPs in contaminated sites.

2. Materials and methods

2.1. Development of analytical workflow for the selected study site

The study area Eloor-Edayar industrial POPs hotspot, which spans an area of approximately 11.2 km^2 and located between 76.29250°–76.30880° E (longitude) and 10.08100°-10.07720° N (latitude), is the only Comprehensive Environmental Performance Indexed (CEPI) area in Kerala, a southern state in India. The region is home to more than 247 small and medium-sized industries, including chemical, petrochemical, pesticide, leather, and fertilizer manufacturing units. Initially, a comprehensive background study was conducted as part of the development of analytical work flow, to understand potential contamination sources, previous investigations conducted, and baseline data from stakeholders. Subsequently, sediments, a natural sink for pollutants, and fish samples were selected as ideal markers to investigate the enviro-food-health risk associated with dioxin like Persistent Organic Pollutants (dl-POPs) in riverine ecosystems. Moreover, the number of fish species in the Periyar River has dropped from 35 (as in 1980) to 12 due to severe industrial pollution and deterioration in water quality (Ambily and Menon, 2019; Ramachandra et al., 2012). Fish samples from Vellayani Lake in Thiruvananthapuram, Kerala, which is located in a non-industrial region, were taken as the field control site due to reports stating relatively low pollution (Das et al., 2017) while those from the open market were used as a market control sample. Further, matrix-specific analytical steps such as extraction, clean up, and quantification, were developed for sediment/fish samples and validated in accordance with regulatory requirements. It was then employed to generate dl-POPs levels in samples from the above mentioned sites and was processed by statistical and bio-monitoring tools to elucidate critical parameters that represent the hotspot's current status.

2.2. Sampling

In this study, a total of 72 fish samples (each species, three samples per sampling site) and 18 sediment samples (three samples per sampling site) were collected from the river body surrounding the hotspot. The sampling team identified six specific locations in the river body where local fishermen engage in fishing activities to provide fish for the local community and all these locations around the industrial area were chosen. The selection of these sampling points was intended to assess the health risk to fish consumers without bias. A number of other characteristics were considered in the strategic selection of sampling sites, such as proximity to industrial areas, canals connecting industrial areas and the main flow of the river. Out of the six sampling points, four points (SP1, SP3, SP4 and SP5) in the main flow of the Periyar River and two (SP2 and SP6) from its distributary canal. Four fish species, Etroplus suratensis (*E. suratensis*), Mugil Cephalus (*M.cephalus*), Horabagrus brachysoma (*H.branchysoma*) and *Oreochromis mossambicus* (*O. mossabicus*) were selected for present study. These species were chosen based on their significance in the local community's diet and their abundance in the study area. The fish samples were collected from each sampling point in the study as well as control sites through cast net catching method, while market control samples were procured from the open market. Fish samples with comparable size and weight were selected for analysis to obtain mean contaminant level, specific to each species and sampling point. The corresponding sediment samples were also collected from the locations where fishes were sampled using grab sampler. The trend in the concentration of dl-POPs in the hotspot region vs control site vs general market could reveal the role of source driven studies. All samples were stored at −20 °C until analysis in the laboratory.

2.3. Chemicals and consumables

All certified reference standards used for isotopic dilution calibration and confirmatory analysis such as native and labelled PCDD/Fs, PCBs and Dioxin/PCBs recovery/syringe standards were purchased from Cambridge Isotopic Laboratories (CIL), Massachusetts, USA while labelled non-ortho and mono-ortho PCBs (NO and MO PCBs) were obtained from Wellington Laboratories, Guelph, Canada. Head space GC grade solvents and high purity n-nonane keeper solvent were obtained from Spectrochem, India, and E-Merck, Germany respectively. Multilayer silica, alumina and reversible carbon columns used for sample purification were obtained from Fluid Management Systems (FMS Inc., Waltham, MA, USA). Ultra-high purity helium and nitrogen gas (99.999%, Bhoruka, India) were used as carrier and collision gases respectively.

2.4. Sample preparation and quantification of dioxins and PCBs

The sample preparation and quantification for dl-POPs in fish and sediment samples were carried out at the ISO/IEC 17025:2017 accredited Dioxin Research Laboratory (DRL), CSIR-NIIST. Sediment samples were air-dried, and TOC was determined using a TOC analyzer (3100 multi N/C, Analytikjena, Germany) with an HT 1300 solid module. Homogenized tissue samples were freeze-dried, and sediment samples were grounded into powder before extraction. In a typical experiment, the sample uptake was 10 g for tissue and sediment samples on wet and dry weight basis respectively. Samples were mixed with anhydrous sodium sulphate and spiked with $^{13}C_{12}$ labelled internal standards of all congeners of PCDD/Fs and PCBs and extracted using Accelerated Solvent Extraction (ASE) customized with neutral silica layer assisted inline cleanup step. Lipid content in fish samples was determined as per EC152/2009 (EU 152/2009, 2009). The sample clean-up was carried out using semi-automated EZprep 123 work station (FMS Inc.,USA) which consists of a three-column set up with multi-layer silica, activated carbon and alumina connected in series. The

concentrated extract from ASE (in hexane solvent) was introduced into the clean-up system through sample vial after the conditioning step. The obtained two fractions Fraction 1 (PCDD/Fs & NO-PCBs) and Fraction 2 (MO & non-dioxin like (ndl)-PCBs) were concentrated by nitrogen evaporation (Supervap, FMS Inc, USA) and reconstituted with n-nonane to 200 μL after spiking with recovery standard. The two fractions were then quantified using custom validated GC-MS/MS for dioxins & PCB analysis. The quantification of fraction-1 and fraction-2 was carried out separately using Agilent GC-MS/MS system which comprises of 7890 B gas chromatograph equipped with 7690 A automatic liquid sampler and 7000C triple quadruple mass spectrometer. Effective separation of congeners was achieved using classic DB-5 MS 60 m × 250 μm x 0.25 μm (Agilent) capillary column. The calibration of the instrument and auto tuning was performed biweekly. Optimized extraction, clean-up, and quantification parameters are provided in Table S1.

2.5. GC MS-MS based confirmatory method validation & QA/QC studies

A GC-MS/MS quantification method for fish and sediment samples was validated according to EU regulation 644/2017. Performance criteria, such as calibration efficiency, limit of quantification (LOQ), precision, accuracy, ion ratio, and recovery rates, were optimized (Table S2) using solvent standard calibration method for each congener. Internal and recovery standard concentrations were kept constant in each calibration level. Quantification was carried out by isotopic dilution mass spectrometry, and the R^2 value of each congener was monitored for calibration performance. Internal standard (ISTD) quantification and recovery calculations were based on equations in Table S3 (European Committee for Standardization, 2012) LOQ of each analyte was determined based on a calibration approach following EU Reference laboratory guidance (European Commission, 2016). The developed method was compliant with Toxic Equivalent (TEQ) at LOQ level ≤1/5th of the maximum level (ML) for intended purposes. Precision and accuracy were verified by analyzing spiked blanks at three different levels (ML/2, ML, 2ML) recommended for fish tissue according to EC 1259/201 (Commission Regulation, 2011). The experiments were repeated (n = 8) and calculated intermediate precision in terms of relative standard deviation (RSD %) and the mean value was used to calculate bias% in order to express trueness of the optimized method. The method's efficacy for dl-POPs quantification in sediment samples was verified through participation in the InterCinDIC2021SE proficiency testing program. A representative pork fat matrix was used as quality control material to demonstrate the long-term accuracy, precision, and robustness of the method, as well as to monitor background contamination and cross-contamination in the laboratory. Results were plotted on a quality control plot with mean and mean ± 2 SD boundaries (at 95% confidence level).

2.6. Estimation of bioaccumulation trend and health risk

The Biota Sediment Accumulation Factor (BSAF) is a first level screening tool used by regulatory agencies world-wide to assess the risk to aquatic organisms from organic pollutants and to plan remediation strategies (Burkhard et al., 2004). Fat normalized contaminant level in fish tissue and TOC-normalized sediment levels were used to estimate BSAF values (Table S4), which are specific to fish species and sampling points. Regression analysis of the data was performed using JASP statistical software by determining statistically significant Pearson's correlation coefficients with $p < 0.05$(**Eq 1**). The health risk posed by these contaminants to the fish consumers in the study area vis-à-vis control (field and market) was assessed based on the observed levels in different fish species and the fish consumption pattern stipulated by National Institute of Nutrition, India (NIN, 2020) followed by comparison with the Tolerable Weekly Intake (TWI) recommended by EU (EFSA CONTAM Panel, 2018) to understand the impact of pollution.

$$Pearson's Correlation\ coefficient, r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 (y_i - \bar{y})^2}} \quad (1)$$

$x_i = dioxin\ or\ PCBs\ levels\ in\ sediment$

$\bar{x} = Mean\ value\ of\ dioxin\ or\ PCBs\ levels\ in\ sediment$

$y_i = dioxin\ or\ PCBs\ levels\ in\ fish$

$\bar{y} = Mean\ value\ of\ dioxin\ or\ PCBs\ levels\ in\ fish$

3. Results and discussion

3.1. GC MS-MS based method validation as per EU 644/2017 and QA/QC studies

The validation criteria mentioned in the relevant EU regulations can be broadly divided into three aspects such as basic requirements for confirmatory method, requirements for GC based method and specific requirements for GC-MS/MS based method as given in Table S2 (European Union, 2017). Two precursor ions and two specific product ions for each congener were monitored and the resolution of the mass spectrometer was set to unit resolution. The relative ion intensities were within the specified ±15% tolerance during solvent standard calibration as well as in matrix spike recovery experiments, which proved its adequacy for routine sample analysis. A nine-point calibration curve with excellent linearity for each of the congeners ($R^2 > 0.999$) was obtained for PCDD/Fs and PCBs in the range of 10–2560 pg/mL and 39.06–10000 pg/mL respectively. The lowest acceptable calibration point that meets the analysis criteria namely deviation from average relative response factor (RRF) (<30%), RSD of response factors (±15%), relative ion intensity (±15%) and valid retention time window was used to determine the limit of quantification. The limit of quantification (LOQ) (in pg/g unit) estimated for PCDD/Fs and PCBs by taking into account the sample size of 10 g and the final reconstitution volume of 200 μL were complying with the criteria of ≤1/5th of the ML set forth by EU regulations (Commission Regulation, 2011) for fish and fish products viz. 0.35 pgTEQ/g (PCDD/Fs), 0.39 pgTEQ/g (PCDD/Fs + dl-PCBs), and 13.6 pg/g (ndl-PCBs), and are given in Table S5.

A series of experiments were conducted to optimize the sample preparation procedures (extraction/clean up) (Table S1), and ensured the recovery rates of respective ^{13}C labelled internal standards falls within 60–120%. Thus optimized column chromatographic methodology shown superior interference removal efficiency, better noise reduction and gas-chromatographic separation of isomers (<25% peak to peak between 1,2,3,4,7,8- HxCDF and 1,2,3,6,7,8-HxCDF).

The quantification of PCDD/Fs and PCBs in blank matrix showed that most congeners were either not detected or below the limit of detection, which confirmed its suitability for spike recovery studies (Table S6). The fortified samples at different levels (ML/2, ML and 2 ML) were extracted using ASE and removed the co-extracts by multilayer silica, carbon and alumina based semi-automated clean-up system following the procedure given in Table S1. The obtained RSD and bias % values presented in Table 1 were found to be in accordance with EU regulation limits.

The QC chart generated by analysing the levels of PCDD/Fs and PCBs in the QC material (pork fat) at regular intervals for 9 months is given in Figs. S2–S3. The results for all congener groups were within the range of mean ± 2 SD limit. In addition, the **z**-scores of proficiency test for the sediment samples were well within the tolerable limit (z = ± 2) (Fig. S4.). The observed compliance in accordance with the above mentioned critical analytical performance parameters unequivocally demonstrated the efficacy and robustness of the developed GC-MS/MS based confirmatory method.

Based on the performance figures presented above, it is evident that the developed GC-MS/MS-based methodology demonstrates

Table 1
Trueness and intermediate precision study of the developed method.

Spiking level	PCDD/Fs			dl-PCBs			ndl-PCBs		
	Obtained result	Bias %	RSD%	Obtained result	Bias %	RSD%	Obtained result	Bias%	RSD%
ML/2	1.76	0.571	7.12	1.46	−2.67	5.69	56.3	−9.92	9.21
ML	3.36	−4.00	6.23	2.72	−9.33	6.02	114	−8.80	3.54
2ML	6.70	−4.29	4.28	5.77	−3.83	7.57	240	−4.00	7.72

EU Maximum levels for fish and fishery products: PCDD/Fs (3.5 pg TEQ/g ww), PCDD/Fs & dl-PCBs (6.5 pg TEQ/g w.w), ndl PCBs (125 ng/g ww).

comparable efficacy to GC-HRMS in meeting specific regulatory requirements for the confirmatory analysis of dl-POPs. These two technologies employ different approaches. HRMS accelerates ions to equal velocities and uses a magnetic field for differentiation, resulting in superior mass resolution (up to four decimal points) through Selective Ion Monitoring (SIM). On the other hand, GC-MS/MS utilizes its ability to perform multi-stage mass spectrometric filtering through Multiple Reaction Monitoring (MRM) for exceptional selectivity in quantitation. In terms of technical specifications, HRMS should achieve a resolving power of 10,000 (10% valley) at m/z 304.9824 (PFK), while MS/MS should maintain a resolution of 0.7 amu/Da for effective dl-POPs analysis. Both techniques commonly employ ion ratio ±15% windows, with HRMS focusing on molecular fragment ratios (quantifier ion to qualifier confirmatory ion) in accordance with USEPA 1613 B, and MS/MS focusing on precursor ion to product ion ratios (USEPA 1613 B, 1997). These ion ratio measurements serve as quality control measures in both GC-HRMS and GC-MS/MS methodologies, and the validated GC-MS/MS based method consistently meets this stringent criteria. However, it is crucial to ensure efficient sample clean-up to meet stringent regulatory criteria and obtain unbiased results, even with the relaxation in mass resolution. Furthermore, significant advancements in the efficiency of ion sources in MS/MS systems could enhance the sensitivity, while enabling reduced sample size, matrix effects and sample preparation time which could ultimately result in the increase of lifespan of consumables. On the other hand, higher installation and maintenance costs, lack of stability, time-consuming maintenance, and the need for highly trained professionals to operate and maintain the sector HRMS instruments adds to its complexity. The detailed comparison of these two technologies is provided in Table S14.

3.2. Trends of PCDD/Fs and PCBs in sediment samples

The levels of PCDD/Fs in sediment samples varied between 6.78 and 35.6 pgTEQ/g dry weight (d.w.) across different sampling sites. It can be observed that the most toxic congener 2378-TCDD made the major contribution to the total TEQ of PCDD/Fs at all six sampling points followed by 2378-TCDF and 123789-HxCDD. On the contrary, the absolute concentration of OCDD showed higher values in all sediment samples, ranging from 194 to 12114 pg/g dry weight. This observation is in line with congener finger print data from several studies which is commonly referred to as the "OCDD anomaly" (Kim et al., 2009; Nieuwoudt et al., 2009; Ren et al., 2009; Ssebugere et al., 2013). Although the entire mechanism is still not clear, the prevalence of poor combustion conditions and higher chlorine content favors the formation of kinetically feasible higher chlorinated congeners such as OCDD (Stanmore, 2004). In addition, the poor water solubility of OCDD and its greater affinity to fine particles may lead to long-term accumulation, particularly in organic-rich sediment compared to lower chlorinated congeners (Nunes et al., 2014; Van Thuong et al., 2015). Calculated ratios of PCDFs to PCDDs were <1 in most sediment samples analyzed, suggesting that the formation pathway is via precursor route rather than de-novo synthesis (Huang and Buekens, 1995). There is a very high probability of emission of chlorinated precursor species from surrounding industrial area that may trigger/lead to the formation of PCDD/Fs (Divya et al., 2021). The calculated ∑PCDD/Fs for sediment samples in the present study were higher compared to the values reported in some of the major rivers around the world indicating the role of critical industrial sources in the region (**Table S7**). The sampling points SP1 and SP6 closer to the industrial area (16–30 m) showed higher levels of contamination than SP2 to SP4 which are relatively far away from the industrial region (440–1150 m).

In most samples analyzed, the absolute concentration of PCB 77 and PCB 81 was found to be high and in line with reported values in the Nile and Han Rivers (El-kady et al., 2007; Kim et al., 2009). A strong positive correlation between levels of NO-PCBs and PCDD/Fs was observed at SP6 and SP4, which indicates the increased emission of dioxin and dl-PCBs in these regions from the surrounding industrial area. It was observed that the concentration of higher chlorinated congeners (PCB 156,167,157 and 189) dominated in the analyzed samples due to their lower degradation potential in the environment and organisms (Lavandier et al., 2013). The combined toxic equivalence for dl-PCBs ranged from 2.66 to 8.12 pgTEQ/g dw in the analyzed sediment samples. A two-fold increase in the concentration of dl-PCBs can be observed vis-à-vis studies conducted elsewhere such as in Vaal River (South Africa) and Upper Scheldt River (Belgium-France) (Nieuwoudt et al., 2009a; Sanctorum et al., 2011).

A similar trend in abundance of the more chlorinated congeners PCB 153, 138 and 180 towards the ∑ndl-PCBs concentration can be noticed. Sampling sites SP6 and SP4 near the industrial area (within 500 m) showed relatively higher values of ∑ndl-PCBs which are comparable to the levels reported in Lake Qarun of Egypt (Barakat et al., 2013; El-kady et al., 2007; Ssebugere et al., 2014). The group wise cumulative concentrations of contaminants in analyzed sediment samples are given in Table 2. Congener fingerprints of contaminants in the analyzed sediment samples and the percentage contribution of individual congeners to the cumulative TEQ are given in Table S8, Fig. S5 and S6 respectively.

3.3. Trends of PCDD/Fs & PCBs in fish samples

The average fat content (w/w%) in the analyzed samples was *E. suratensis* (2.36%), *M. cephalus* (5.06%), *H. branchysoma (2.70%)* and *O. mossabicus (1.97%)*. Among the different fish species analyzed, higher levels were observed in *E. suratensis* and *M. cephalus*. The PCDD/Fs values obtained were higher compared to the studies carried out in *E. suratensis* species from the coast of Sri Lanka where the average ∑ PCDD/Fs TEQ value was 0.19 pgTEQ/g and *M. Cephalus* (0.18 pgTEQ/g) in northwest Florida (Guruge and Tanabe, 2004; Karouna-Renier et al., 2011) respectively. The observed concentrations of PCDD/Fs in *E. suratensis* and *M. cephalus* at SP6 and SP4 exceeded/approach the EU regulation maximum level ie. 3.5 pg TEQ/g w. w. These locations are closer to the industrial belt and the corresponding higher concentrations observed in sediment samples, strongly indicate the transfer of dl-POPs from the sediment into fish tissue. 2378 TCDD, 23478 PeCDF and 12378 PeCDD congeners registered the largest contribution to the ∑PCDD/Fs TEQ in most analyzed samples of *E. suratensis* while 2378-TCDF and 12378-PeCDD in the case of *M. cephalus.* Levels of PCDD/Fs in *H. branchysoma,* commonly referred to as yellow catfish, were similar to levels reported in several water bodies in southern Mississippi, USA where the concentration varied between 0.09 and 3.14 pgTEQ/g (Scott et al., 2009). In most *H. branchysoma* samples analyzed, 2378-TCDD, 12378-PeCDD and 23478-PeCDF congeners made the largest contribution to the total TEQ. In addition, it has been observed that *O. mossabicus*

Table 2
Average pollutant concentrations in fish and sediment samples collected at different sampling sites in the study area.

Sample	Category	SP1	SP2	SP3	SP4	SP5	SP6
Sediment	∑PCDD/Fs	23.2 ± 2.01	9.25 ± 0.931	6.78 ± 0.953	29.8 ± 3.02	12.3 ± 1.07	35.5 ± 1.83
	∑dl-PCBs	6.07 ± 1.03	3.45 ± 0.0652	2.66 ± 0.179	6.62 ± 2.03	2.86 ± 0.0756	8.12 ± 1.19
	∑ndl-PCBs	83.7 ± 7.92	45.0 ± 5.02	34.2 ± 3.02	103 ± 10.1	24.7 ± 2.31	100 ± 11.2
E. suratensis	∑PCDD/Fs	2.16 ± 0.193	1.72 ± 0.00453	1.33 ± 0.0956	3.08 ± 0.984	1.82 ± 0.0521	3.47 ± 0.856
	∑dl-PCBs	0.972 ± 0.0162	0.580 ± 0.00124	0.560 ± 0.00213	0.820 ± 0.120	0.421 ± 0.0325	1.78 ± 0.0425
	∑ndl-PCBs	6.58 ± 1.27	4.29 ± 1.02	3.60 ± 0.0561	9.42 ± 1.72	2.78 ± 0.0142	6.94 ± 0.794
M.cephalus	∑PCDD/Fs	3.16 ± 1.35	2.72 ± 0.0632	1.21 ± 0.0321	3.12 ± 0.562	2.90 ± 0.0156	4.03 ± 0.721
	∑dl-PCBs	1.18 ± 0.0632	1.10 ± 0.106	0.104 ± 0.00523	1.13 ± 0.195	0.0816 ± 0.00122	2.13 ± 0.132
	∑ndl-PCBs	8.40 ± 1.12	5.00 ± 0.856	5.36 ± 1.20	10.1 ± 1.93	2.54 ± 0.0425	10.9 ± 1.11
H. branchisoma	∑PCDD/Fs	2.20 ± 0.0853	1.29 ± 0.00235	0.86 ± 0.00356	2.37 ± 0.042	1.53 ± 0.0369	1.97 ± 0.0523
	∑dl-PCBs	0.203 ± 0.0123	0.103 ± 0.00425	0.101 ± 0.00530	0.240 ± 0.00212	0.118 ± 0.00153	0.235 ± 0.00125
	∑ndl-PCBs	4.04 ± 0.923	2.77 ± 0.326	1.60 ± 0.0231	6.52 ± 0.896	1.43 ± 0.0268	9.95 ± 1.22
O.mossambicus	∑PCDD/Fs	0.789 ± 0.00256	0.648 ± 0.0512	0.518 ± 0.00421	0.911 ± 0.0125	0.762 ± 0.00142	0.884 ± 0.00742
	∑dl-PCBs	0.203 ± 0.0253	0.136 ± 0.00921	0.160 ± 0.00329	0.186 ± 0.00425	0.109 ± 0.00521	0.239 ± 0.00896
	∑ndl-PCBs	2.40 ± 0.0756	1.51 ± 0.0246	1.45 ± 0.00142	3.56 ± 0.523	1.24 ± 0.00931	2.29 ± 0.0108

Units: ∑PCDD/Fs (pgTEQ/g), ∑dl-PCBs (pgTEQ/g), ∑ndl-PCBs (ng/g).
Weight basis: Sediment (dry weight basis) Fish tissue (wet weight basis).

species have the lowest PCDD/Fs contamination, presumably due to lower fat content. Such discrepancies between intra/inter species attributed to different dietary habits and trophic level as reported elsewhere (Van der Oost et al., 2003). It may be noted that the benthic feeding *M. cephalus* showed a higher tendency to accumulate, leading to increased PCDD/Fs and dl-PCBs levels in most samples. The levels of dl-PCBs found in O. *mossabicus* and H. *branchysoma* were comparable and small variation were only observed between different sampling sites, in contrast to wide variation observed in *E. suratensis* and *M. cephalus* species. Bottom dwelling fish species such as *E. suratensis* and *M. cephalus* sampled from SP6 exhibited higher ∑ PCDD/Fs & dl-PCBs, indicating possible higher contamination with dl-POPs at SP6.

The higher chlorinated ndl-PCB congeners such as 153, 138 and 180 dominated in all the examined fish samples similar to that in sediment samples, due to their low degradation potency. The group wise contaminant levels, congener finger prints and the percentage composition of the individual congeners towards the respective group-wise cumulative account are given in Table 2, Tables S9–S12 and Figs. S7–S11 respectively. Large interspecies differences in pollutant concentrations were observed even in samples taken from the same sampling point. This can be attributed to a number of factors such as difference in dietary habits, food intake, lipid content and biochemical reactions in the organism. Furthermore species-specific differences in metabolism, detoxification mechanisms, and excretion processes can result in varying rates of pollutant absorption, metabolism, and elimination (Barron, 1990; Ssebugere et al., 2013). These possible variations does not significantly affect the developed work flow, intended to study extend of dl-POPs contamination and health risk estimations at the hotspot region. Control samples from both non-industrial area and open market showed lower levels vis-a-vis hotspot, which emphasized a potential risk of POPs contamination in the study region due to industrial discharges and poor waste management and could pose long term health risks to the fish consumers. Detailed discussion regarding analysis of control samples is given in **Section 1** and Fig. S12 of supplementary material.

3.4. Comparative evaluation of present status of hotspot vis-à-vis global scenario

A thorough examination of prior research and reports relating to the POPs hotspot revealed that certain incidents/evidences of harmful effects of pollution in this area have been reported such as frequent fish deaths and deterioration in air and water quality (Anjusha et al., 2020; Suchitra, 2020; Charuvilayil, 2013). Majority of the research carried out in the area was focused on contamination due to pesticide-based POPs such as DDT, Dicofol etc, while only a few studies reported on dl-POPs (Deepa et al., 2008; Stringer, 2003; Sujatha et al., 1994). As part of the global dioxin surveillance project, IPEN reported the only available study in the hotspot region, where 4 times higher levels of dioxins (13.91 pg TEQ/g fat) and PCDD/F/dl-PCBs (15.08 pg TEQ/g fat) in eggs of free-range chickens than EU regulations were observed (Petrlik et al., 2005). This study also indicated that the congener profiles observed in the egg samples were similar to those obtained from samples taken from an outdated pesticide stockpile in Vikuge, Tanzania, confirming the possibility that pesticide production facilities could serve as sources of dioxins. Fig. 1 depicts the peak total TEQ/concentration of congener groups (**(i)** PCDD/Fs, **(ii)** dl-PCBs, **(iii)** ndl-PCBs) observed in sediment (**1 (a)**) and fish (**1(b)**) samples from the different sampling points. The levels of contaminants (∑ PCDD/Fs + dl-PCBs) in sediment samples taken from three sampling points (SP1, SP6, SP4) exceeded the action level of 21.5 pgTEQ/g recommended by Canada for eco-toxicological assessment. In contrast, the levels were well below the remedial action level set by Japan (150 pgTEQ/g) and the Netherlands (1000 pgTEQ/g) (Suzuki et al., 2016) based on human risks. Fish and sediment samples taken from SP6 showed higher concentrations of all three categories of dl- POPs. The site SP6 is located in close proximity to the industrial zone, indicating a strong confluence of emission source - environmental - food nexus towards bioaccumulation. The cumulative PCDD/Fs and dl-PCBs levels varied between 0.68 and 6.16 pgTEQ/g wet weight in the analyzed fish samples from different sampling points, and were below the EU maximum of 6.5 pg TEQ/g wet weight. Although ∑ dl-POPs were not alarming, the levels of dioxins obtained were higher/equal in *M. cephalus* and E. suratensis than the EU maximum level of 3.5 pg TEQ/g for fishery products. The limited surveillance of dl-POPs in this region may be due to inadequate facilities and cost effective validated analytical methods. From this perspective, the developed analytical workflow can serve as an excellent tool for enabling low-cost routine monitoring of POPs hotspots worldwide.

Fig. (1c) depicts a comparison plot of sediment levels in the current hot spot under investigation with other hot spots around the world. Although the IPEN identified forty hot spots of Persistent Organic Pollutants (POPs) globally, surveillance studies were constrained. The selection of these POPs hot spots was based on the presence of various potential emission sources of POPs in these areas and limited number of sample analyses. Among the available studies, the majority focused on the most toxic congener group dioxin and furans while PCBs are less studied. The highest reported levels of POPs were found in the Pacer Ivy area of Vietnam (1.50×10^3 pgTEQ/g) due to historical Agent Orange usage. In comparison to the limited global data available, the extent of contamination at the present hot spot is moderate. The scarcity of surveillance data underscores the urgency of developing viable analytical methods for regular monitoring of these hot spots to ensure human

health security.

3.5. Correlation analysis and bio accumulation trends of contaminants

The benthopelagic nature of the fish species analyzed in this study could result in the long-term accumulation of sediment-bound contaminants in their tissues. To investigate possible correlations, regression analysis was performed by estimating Pearson's correlation coefficient between the levels of PCDD/Fs, dl-PCBs (WHO2005 TEQs), and ∑ndl-PCBs in fish species and the corresponding sediment levels at each sampling point. The regression analysis showed a strong overall positive correlation between the contaminant levels in the analyzed fish and sediment samples, regardless of the contaminant group/subgroup, as evidenced by various measurable indices such as correlation coefficients and statistical significance ($p < 0.05$) (Fig. S13.). This positive correlation suggests high bioaccumulation of these contaminants from sediment into fish tissues, which is consistent with a similar correlation observed between mussel fish and sediment samples from Kentucky Lake, USA (Loganathan et al., 2008). Approximately 83.3% of the regression analysis data were statistically significant with a p-value <0.05. These findings can be used as a preliminary tool to assess the tendency for bioaccumulation of contaminants from sediment into fish. Furthermore, to elucidate congener/species-related bioaccumulation trends, a more in-depth study was conducted by estimating Biota Sediment Accumulation Factor (BSAF) using absolute concentration of contaminant groups instead of levels expressed in terms of TEQ. The TOC levels varied between 0.011 and 0.019 g/g dw for sediment samples. The estimated BSAF values with absolute concentrations of individual congeners shown that higher order bio-magnification of dl-PCBs occurred in all fish species vis-à-vis PCDD/Fs and ndl- PCBs (Fig. 2.). It also demonstrated the crucial role of BSAF studies in identifying the underlying mechanistic pathways involved in congener wise accumulation trends and their overall impact on toxicological aspects. The degree of chlorination and the position in the aromatic ring together with other physicochemical properties determine the bioaccumulation tendencies of dl-POPs congeners (Stohs, 2014). PCB congeners with high chlorine substitution as well as lower ortho substitution have poor volatility and water solubility, resulting in higher affinity for sediments (IARC, 2015). For PCDD/Fs, BSAF values ranged from 0.019 to 0.092. Compared to other sampling points higher BSAF values for PCDD/Fs were observed in SP2 for all analyzed fish species. Samples of *M. cephalus* (0.0920) and *H. barchysoma* (0.0810) from SP2 showed higher PCDD/Fs-BSAF values among the fish samples analyzed in the present study. Higher BSAF values at SP2 can be attributed to the highest TOC level observed in the sediment samples from SP2 (0.019 g/g dw of sediment), compared to the other sampling points. The low BSAF values observed in highly 0 (SP4), 0.033 (SP5) and 0.038 (SP6), which reiterated the critical role of bioavailability, hydrophobicity and feeding pattern in understanding the accumulation pattern (Lake et al., 1990). For dl-PCBs the calculated BSAF values ranged from 4.00×10^{-3} to 0.671. In addition, higher BSAF for ndl-PCBs were observed in *E. suratensis* at all sampling points ranging from 0.0180 to 0.0710. The BSAF values obtained indicated a higher accumulation of PCDD/Fs in *O. mossabicus* and *M. cephalus* and dl and ndl-PCBs in *E. suratensis* species, suggesting the need for detailed studies to understand the factors contributing to the trends in bioaccumulation such as contaminant metabolic rates and feeding nature.

3.6. Health risk assessment of fish consumers in the study area

The health risk assessment for fish consumers in the study region was performed by calculating the combined dietary intakes of PCDD/Fs/dl-PCBs. WHO recommended a tolerable daily intake (TDI) of 1–4 pgTEQ kg^{-1} body weight (bw) day^{-1}, while EFSA recently suggested a more cautious tolerable weekly intake (TWI) level of 2 pgTEQ kg^{-1} bw $week^{-1}$ (Van Leeuwen et al., 2000; EFSA CONTAM Panel, 2018) The

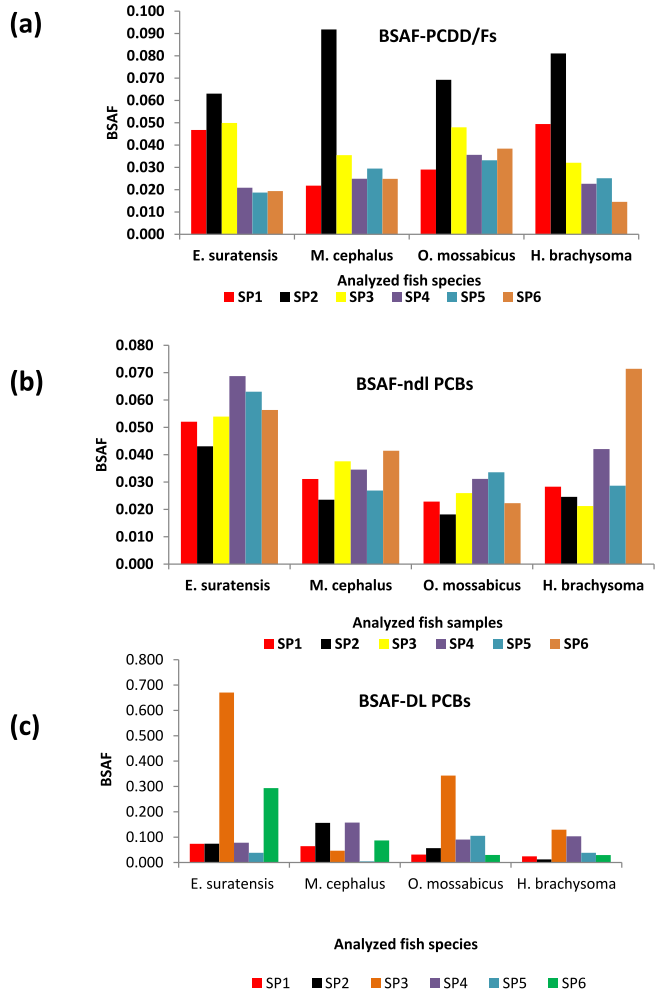

Fig. 2. Biotata sediment accumulation factors for different fish species (a) BSAF PCDD/Fs, (b) BSAF dl - PCBs and (c) BSAF ndl- PCBs.

daily fish consumption in rural South India is 80.7 g and the average adult bodyweight is 60 kg which has been taken into account for the estimation of the species-specific daily intake of fish consumers (NIN, 2020).

The calculated daily intake varied between 0.89 and 8.41 pgTEQ kg^{-1} bw^{-1} across different fish species and sampling locations. A comparative analysis of the results of the present study vis-à-vis WHO

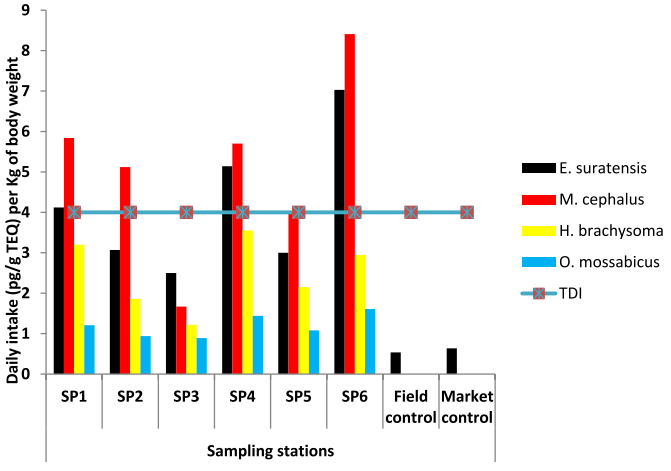

Fig. 3. Health risk assessment to fish consumers in the study area.

recommended higher TDI value (4 pg TEQ kg^{-1} bw day^{-1}), (Fig. 3.), suggested the likelihood of an increased risk for consumers in the case of *M. cephalus* and *E. suratensis*. Consumption of *M. cephalus* could result in the daily intake levels from 1.67 to 8.41 pgTEQ kg^{-1} bw day^{-1}. Four out of six sampling sites reported the possibility of a higher daily intake compared to TDI in the case of *M. cephalus*. Sampling points SP1, SP4 and SP6 reported higher daily intake for *E. suratensis* and values ranged from 4.12 to 7.03. Despite differences in fish species and sampling sites, the calculated weekly intake values for *E. suratensis* (17.5–49.2), *M. cephalus* (11.7–58.9), *H. brachysoma* (8.54–24.9), and *O. mossabicus* (6.23–11.3) were observed to exceed the more conservative EFSA TWI limit of 2 pgTEQ kg^{-1} bw $week^{-1}$. The weekly intake values in Baltic fish species reported elsewhere were 8–24 times higher compared to the EFSA assigned TWI and is comparable to the present study (Mikolajczyk et al., 2021). In contrast, the daily intake associated with Mediterranean swordfish was found to be relatively low compared to the present study ranging from 0.910 to 3.15 (Mehouel et al., 2021). Overall, these findings suggest a potential health risk for consumers of fish in the POPs hotspot region of global importance, and highlight the need for regular monitoring with affordable confirmatory tools, establishment of consumption guidelines, and scientific pollution control and remediation measures.

4. Conclusions

In conclusion, this study presents a cost-effective integrated workflow for assessing the environmental and health risks associated with dioxin-like Persistent Organic Pollutants (dl-POPs) in industrial hotspot regions. The developed GC-MS/MS-based analytical methodology, validated according to EU regulation 644/2017, demonstrates high accuracy, selectivity, and precision. The study provides valuable insights into the prevalence and sources of dl-POPs in the ecosystem based on the studies in fish and sediment samples from a significant dl-POPs hotspot, the Eloor-Edayar industrial belt in India. The findings reveal that dl-POPs are primarily formed through precursor pathways, indicating the potential release of chlorinated precursor species from the surrounding industrial area. Fish samples from the hotspots exhibit significantly elevated levels of polychlorinated dibenzo-p-dioxin/furans (PCDD/Fs) and polychlorinated biphenyls (PCBs) compared to control sites, underscoring the importance of regular surveillance in hotspot regions. Correlation analysis supports the transfer of contaminants into the food chain, further emphasizing the need for ongoing monitoring efforts. Furthermore, the estimated weekly intake of dl-POPs from fish consumption in the study region exceeds the maximum levels established by the European food safety authority, suggesting a potential health risk for fish consumers. Consequently, it is crucial to implement periodic surveillance using user-friendly and validated confirmatory tools to safeguard both human health and the environment. The identified congener fingerprints, correlation patterns, and source attribution of dl-POPs serve as valuable metadata for understanding the fate of these compounds in the environment and developing effective mitigation strategies. Looking ahead, this study paves the way for future advancements in dl-POPs research. Expanding the application of the GC-MS/MS-based workflow to other matrices and emission sites would enable a comprehensive assessment of the environmental-food-human health nexus and support the stakeholders to make informed decisions and devise global strategies to mitigate the risks associated with dl-POPs. Ultimately, the integration of cost-effective analytical methodologies and field-deployable workflows will contribute to the efficient monitoring and management of dl-POPs, ensuring a safer and healthier environment for present and future generations.

Credit author statement

Mr. Kirankumar P S: Analytical Method Development, Experimental studies, Investigation, Data generation, manuscript writing and finalization. Mr. Sanath K: Sampling, GC-MS/MS Analytical method development, Optimisation. Mr. S V Ajay: Sampling, Manuscript drafting, Correlation studies, Interpretation. Ms. Amala Varghese: Sample preparation & Quality control/assurance. *Dr. K. P. Prathish: Conceptualisation, Experimental & Analytical Supervision, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122161.

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