UPO

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SCHOOL OF MEDICINE
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MASTER'S DEGREE IN MEDICAL BIOTECHNOLOGIES

Experimental Thesis on

Exposome investigation in healthy subject using an untargeted metabolomic approach

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1 Summary

1.1 The rationale of the Study

The scientific community is in broad agreement that genetic and environmental factors, specifically chemical pollutants, nutrition, lifestyle, and stress are strongly interlinked in a very complex way, leading to diseases that cause the greatest burden on public health in society. Focusing on environmental factors for epidemiological studies will be an appealing approach to these problems, particularly if at-risk subgroups can be found. However, to understand this, we are limited in our precise knowledge of how everyone will respond to exposure that leads to the development of disease. It would be therefore useful to create a baseline for the evaluation of the impact of exposure at the individual level by considering the most crucial characteristics to differentiate. In the present study, we investigated the role of exopsomes based on age and gender.

1.2 Planning of the Study

Serum samples were collected from healthy volunteers exposed to the same working environment (hospital). Samples were stratified based on age (Young, Middle, and old) and sex. Exposome molecules were extracted from samples and then untargeted analysis was performed with GC×GC-TOFMS to identify and quantify them.

1.3 Results

Through an untargeted study, 66 exposomes in total were successfully identified. There was not much difference found in the sex variables. Exposomes were classified into nine categories. Exposome's category-wise classification offers useful information and stratification. significant exposure to pesticides, PCPs, industrial chemicals, and household chemicals was noted. Both sexes in their middle years have significant exposure to the household category. Similar exposure to PCPs was noted in both genders across all age categories.

1.4 Discussion

The study offers an innovative way to examine exposomes using untargeted analysis. Since age is a key factor that influences variance in the contribution of exposure to specific factors in human health, it is necessary to take age groups of both genders into account in order to better understand the characteristics of human exposure.

2   Introduction

In recent years, the investigation of environmental factors impact on human health drastically thrived, and has turned into a novel emerging field called "exposomics".[1]

Exposome term first coined by Dr. Christopher Wild in 2005, reflects the cumulative measure of surrounding conditions and their relationship with biological responses throughout an individual's lifespan, from birth to death. To understand exposomes in depth there is a need to consider two main things, primarily the nature of these exposomes and secondarily fluctuations that occur over time. According to Dr. Wild, exposomes can be categorized into three main non-genetic exposure domains: internal, specific external, and general external.[1]
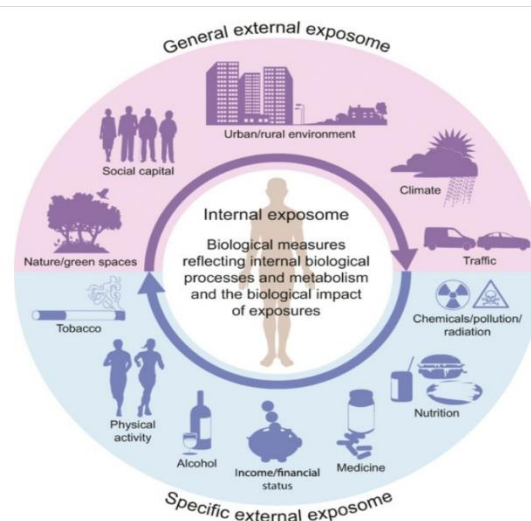
Figure 2.1 Vital domains of the exposome[1]

Regarding the first domain, the exposomes that are in co-relation with internal processes occur in the human body like metabolic functions, endogenous hormone levels, body morphology, physical activity, gut microbiota, inflammatory responses, lipid peroxidation, oxidative stress, and aging. All these processes are cumulatively considered an endogenous factor that has a strong effect on the cellular environment. Secondly, specific external events include radiation, infectious agents, chemical contaminants, environmental pollutants, diet, lifestyle factors such as tobacco and alcohol use, occupational hazards, and medical interventions. Formerly, these factors were the center of attraction for epidemiological studies which enabled the relationship between environmental risk factors and cancer. Thirdly, the general exposure includes societal, economic, and psychological factors that influence individuals, such as social capital, educational attainment, financial standing, psychological stress, urban or rural living environments, and climatic conditions.

When combining all three main domains provides a detailed view of the exposomes which not only focus on physiological processes but also external vital factors like pollutants, and the socio-ecological, and cultural aspects from which an individual has originated. Further Dr. Wild categorized the total exposure profile over time into major topics,

Inherent Composition of the Exposome: concerned about internal physiological processes, specific external agents, and broader social, cultural, and ecological contexts.

Biological Consequences: mainly targets the effect of the previously mentioned category on molecular, cellular, and systemic functions along with their role in the pathogenesis of diseases.

Methodology for Measurement: commenting on investigation methods, involves a challenge to develop a sensitive method for quantifying and analysing accurately the targeted molecules considered as exposomes. This can be done by including some assessments like biomonitoring, environmental sensing, and data integration developments which ultimately ensure a precise and thorough analysis.

Hence to conclude the necessity of exposomic advancement and the enlightening effects of exposome on health, their analysis by robust and actionable techniques development, several projects have been conducted by the European Union (EU) including:

- **HELIX:** Demonstrates how early life exposures impacted health outcomes by considering the mother-child cohort.
- **HEALS:** Multiple cohorts are included such as mother-child cohort, adults/general population, and children provide information on the combined effects of environmental and lifestyle factors on health.
- **EXPOsOMICS:** This study is crucial for long-term studies incorporating cohorts like mother-child cohorts, newborns, and adults which broadly helps to understand the relationship between environmental risk factors and the health through life stages.
- **HBM4EU:** For this study, human biomonitoring development advancement to access exposure to chemicals and their health effects is the key goal established by considering the mother-child cohort.[2]

The significance of these studies with various population-based exposome research offers a complete framework for appreciating the complex nature of the exposome and its effect on human health.

Factoring in complex features and the continuously evolving nature of the exposome, accurate and precise characterization is of utmost importance to verify the co-relation with human health. Under this requirement, metabolome analysis will be prioritized to enhance knowledge about exposome. Metabolome encompasses vivid small molecules with mass below 1000 Da that undergo some chemical modifications as a part of the metabolism process. As a result, the end product of various cell processes represents the cell function. Hence it is a key thing, to begin with, that the metabolome concept majorly understood by metabolomic, offers both qualitative and quantitative insights into the output cellular regulatory pathways that comprise both metabolites and metabolome. Metabolomics plays a crucial role not only by providing valuable data on the biochemical characteristics of living organisms but also information about pathological conditions that arise from metabolic alterations, some genetic modifications, or hazardous environmental exposure.
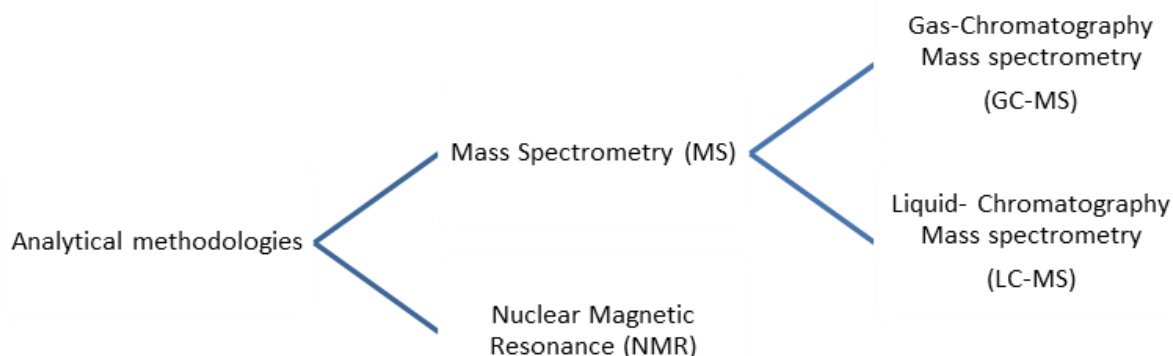
Figure 2.2: Analytical techniques primarily involved in exposome analysis

Several analytical methodologies come under metabolomic investigation, but the main two approaches are nuclear magnetic resonance (NMR) and mass spectrometry (MS), often coupled with either gas chromatography (GC-MS) or liquid chromatography (LC-MS) to facilitate accurate identification and quantification of metabolites. When it comes to sensitivity, MS is the preferred tool for metabolomics over NMR. Within the metabolome, there is a very minute presence of metabolites that are hard enough to detect and quantify but possess significant phenotypic effects. With the help of a predefined library or database, MS permits the identification of any metabolite even in a lower concentration unless a specific method has been applied. The calibration method is an integral part of the metabolomic analysis which enhances the quantification accuracy in mass spectrometry. Moreover, when it comes to diversity concerning chemical compounds across a wide range, expresses specificity, especially by coupling with GC or LC.[3] About exposome, the leading challenge is to capture an extensive range of concentrations within the human body. As compared with endogenous metabolites, medications, and biomarkers from food concentration in blood samples, environmental pollutants depict over ten orders of magnitude. To tackle this, coupling with GC or LC is considered the first approach. Additionally, a second approach involves targeted analysis which specifically focuses on metabolites even in the lower concentrations of exposome compounds. GC-MS analysis is a potential tool for exposomic research as it enhances the separation of complex mixtures of metabolites while considering the properties of environmental chemicals such as hydrophobic, semi-volatile to volatile, and poorly ionized using LC-MS methods, all together complementing the sensitivity and specificity of exposome metabolites. The analysis comprises the universally applicable retention-time indices and highly consistent spectra which makes the method more reliable. The targeted analysis is used to quantify selected molecules that may have a highly significant impact, in terms of toxicity while 'untargeted analysis' used to investigate unknown targets and to perform comprehensive studies. Recently, the GC-MS approach has been developed in the exposome era to identify novel biomarkers, evaluate environmental pollutants, and mainly understand this exposure with a human lifestyle to demonstrate an association with environmental risk factors and diseases. Accessing a broad range of biomatrix such as serum, whole blood, plasma, urine, nails, hair, human milk, saliva, placenta, and exhaled breath condensate for analysis plays a crucial role in the analysis. However, most of the investigations have been focused on specific pollutants and their involvement in disease progression. Considering this

strategy, it helps in understanding the molecular biology of disease despite that will not be able to focus on other parameters responsible for the disease. Hence to analyze extensively, a biological sample, an untargeted analysis is often necessary.[4–6]

Exposome profiles can be widely accessible by considering exposure during critical periods of development. To fulfill this requirement most of the studies involved exposome analysis by focusing on parental exposure, maternal and cord blood sample analysis (prenatal exposome), and mother-child cohort, newborn, and adult population cohorts. This will assist in knowing the importance of parental exposure to children's development or lifestyle in the early stage. Also during counteracting different phases of life, it will be useful to characterize the exposure to understand its contribution to disease development.[7]

Age and sex are the most important variables that need to be considered as they result in variations of outcome when interacting with environmental exposure. Focusing on the human age will predominantly explain the diversity of exposure as it broadly covers the critical phases of life from birth to death. Comparative co-relation demonstrates the stage of life likely prone to disease development due to interaction with environmental risk factors. On the subject of exposome evaluation based on sex, in medicine or public health research often there is a sex bias observed. Though it's proven that there are systematic differences between males and females which shift effect on health as per the exposure even then sex diversity has been overlooked for exposomic research purposes. For instance, females are more in use of cosmetic products, some menstrual and intimate care products which come under personal care products (PCPs) as compared to males. This resulted in higher chemical exposure to carcinogens, metals, endocrine-disrupting chemicals (EDCs), nanoparticles, etc. Depending upon each category of exposure, health status is impacted. Hence it is a crucial part of exposome characterization in terms of sex to thrive in human health.[8]

To sum up, recent advances in exposome-level analysis encouraged the depth of environmental factors' contribution to human health status based on targeted evaluation. Nonetheless, how environmental exposure consequences will vary when it comes to the diversity in age and sex is still not explored.

The objective of this study was to investigate the exposome in healthy subjects exposed to the same working environment. To this aim, we used an untargeted metabolomic approach with bidimensional gas chromatography coupled with mass spectrometry (GC×GC-MS) to analyze serum samples from 57 subjects of different age (young, middle-aged, and old persons) and sex.

3 Materials and Methods

3.1 Study Design

Serum samples from an observational cohort study on healthy subjects were used. A total of 57 samples from subjects divided into three groups based on age were analyzed. The three groups were divided as follows: Young (20-35 years old), middle-aged (36-50 years old), and old (51-71 years old). All the subjects were employees at the internal medicine department of Novara University Hospital.

Table 3.1: Characteristics of Healthy volunteers who participated in the study (Age group)

Age groups (Yrs.)	F (n)	F (%)	M (n)	M (%)	Total (n)
21-71 (Range)	**29**	50.87719	**28**	49.12281	**57**
20-35 (Young)	10	52.63158	9	47.36842	19
36-50 (Middle)	9	47.36842	10	52.63158	19
51-71 (Old)	10	52.63158	9	47.36842	19

3.2 Chemicals and reagents

For the analysis, LC-MS grade solvents and reagents were used. Water and acetonitrile were obtained from VWR (Milano, Italy), methanol was from Scharlab (Barcelona, Spain), ACN: MeOH (9:1) and hexane, IS hexadecane and 3,4,5-tri-nitrochloroaniline were from Merk (Darmstad, Germany). Pure nitrogen gas was supplied by Nippon Gases (Milano, Italy). The Chromabond C18 SPE 3ml/500mg cartridges were purchased. To avoid contamination glass vials had used and related plastic materials were avoided. All glassware was pre-heated at 180 oC for 2 h, also all screw caps were sonicated for 10 min by using methanol as solvent.[9]

3.3 Sample collection and preparation for metabolomic analysis

The samples were prepared as previously reported by Xiu Wang et al.[9]
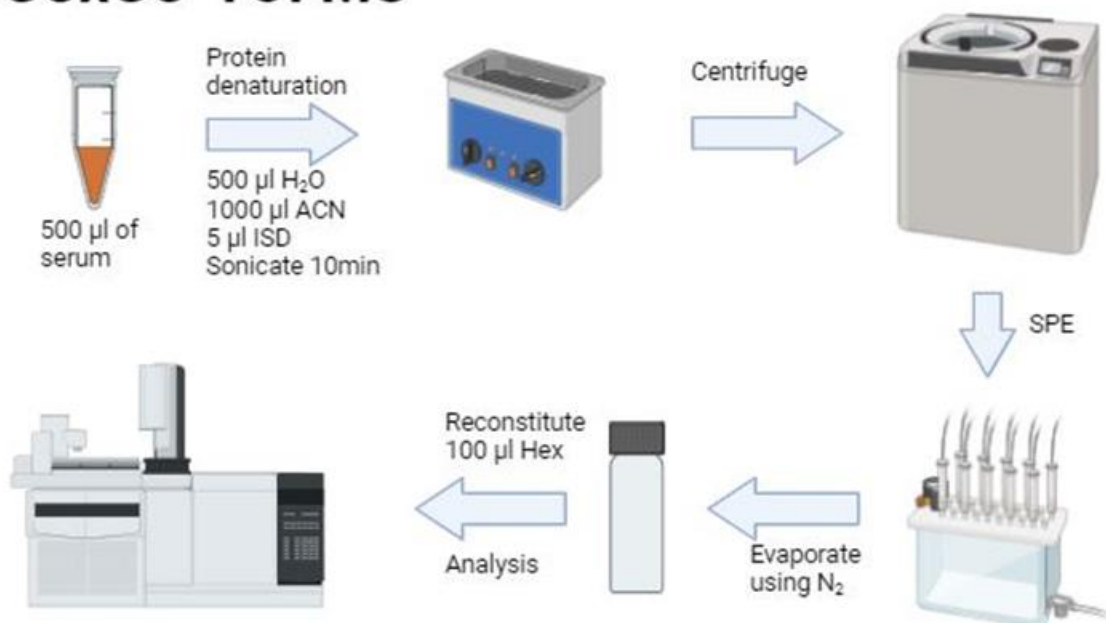
GCxGC-TOFMS

Figure 3.1: Schematic representation for sample preparation to metabolite analysis by GC×GC-TOFMS analysis

1) Protein precipitation:
   - A 500 µL serum sample was diluted with 500 µL water. Addition of 5 µL of 3,4,5-Trichloro-Aniline and 5 µL of Hexadecane were added as an internal standard (IS).
   - Further, 1000 µL of ACN was added. The sample was vortexed and
   - Ultrasonicated the samples in a water bath for 10 min at RT.
   - Centrifugation was performed at 4 °C for 15 minutes with a speed of 21.1000 g.1600 µL of supernatant was collected in a glass vial.
2) Solid Phase Extraction (SPE) on C18 Column:
   - Pre-conditioning of the column- C18 cartridges were washed with 5000 µL methanol and were conditioned with 3000 µL ACN & 5000 µL of water.
   - Loading the sample- 1600 µL serum sample was loaded using vacuum pressure.
   - Rinsing the column- The C18 column was then rinsed and washed with 5000 µL water and then evacuated for about 1 hour to remove residual water.
   - Elution- Finally, the target molecules were eluted with 3000 µL methanol and 3000 µL ACN: methanol (9:1, V/V).
3) The elute was evaporated to near dryness under a gentle nitrogen stream and reconstituted in 100 µL of hexane for GC×GC-TOFMS analysis.
4) Blank samples were also prepared following the same protocol but using LC-MS water.

3.4 GC×GC-TOFMS Analysis

For metabolomic analysis, a LECO Pegasus BT 4D GC×GC-TOFMS instrument (Leco Corp., St. Josef, MI, USA) acquired with a LECO dual-stage quad jet thermal modulator was utilized. The samples were analysed by using the first-dimension column 30 m Rxi-5Sil (Restek Corp., Bellefonte, PA, USA) MS capillary column with an internal diameter of 0.25 mm and a

stationary phase film thickness of 0.25 μm, while the secondary column was a 2 m Rxi-17Sil MS (Restek Corp., Bellefonte, PA, USA) with the same diameter and the thickness as the first. High-purity helium (99.999%) was used as a gas with a flow rate of 1.4 mL/min. Then, 1 μL of the sample was injected at 250 °C in splitless mode. The structure for the temperature flow was, that the initial temperature was set at 45°C for 1 min, then increased by 30°C/min up to 130°C for 3 min, 12°C/min up to 180°C, 7°C/min up to 240°C and maintained at this temperature for 5 min. The secondary column was kept at +5 relative to the GC oven temperature of the first column. The programming rate was the same for both columns. Electron impact ionization was applied (70 eV). The ion source temperature was set at 250°C, and the mass range was 25-550 m/z with an extraction frequency of 32 kHz. The acquisition rates were 200 spectra/s and the modulation period was 4 s for the entire run. The modulator temperature offset was set at +15°C relative to the secondary oven temperature, while the transfer line was set at 280°C.11

The chromatograms were acquired in total ion current (TIC) mode. The peaks with signal-to-noise (S/N) values lower than 500.0 were rejected. ChromaTOFRSync version 2.0 was used for the raw data processing. The mass spectral assignment was performed by matching with NIST MS Search 2.3 libraries. Statistical analysis was performed with metaboanalyst software (www.metaboanalyst.org).

Internal standards (3,4,5-Trichloroaniline and hexadecane) were used to monitor instrument stability and/or data normalization.

4 Results

4.1 GC×GC-MS Findings

This section presents the key findings obtained with metabolomic analysis performed on samples from healthy subjects, these data provide an in-depth examination of the human exposome in subjects exposed to the same working environment. The analysis revealed variations in chemical exposure across age and gender. By utilizing the enhanced separation capabilities and high sensitivity of GC×GC-MS, a detailed profile of environmental contaminants and their distribution patterns were examined. This section highlights the significant variances observed in the exposome and describes how these factors can impact the exposure of healthy individuals.[10]

4.1.1 Identification of Exposome Molecules

After the extraction of exposome molecules from serum samples, chemicals were analyzed using an untargeted approach. Exposome was then identified based on the mass spectra and the identity of molecules was confirmed using mass spectra and retention index. Figure 4.1 shows a chromatogram and Figure (No) describes an example of mass spectra for the identification of molecules.
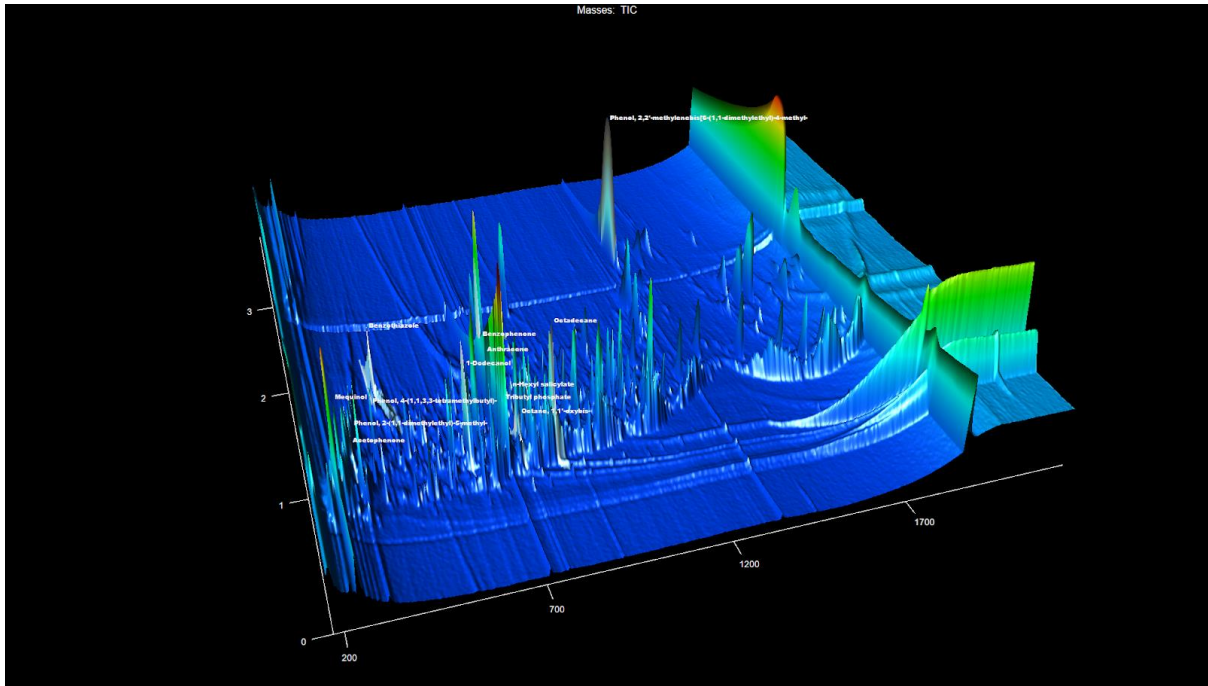
Figure 4.1: 3D chromatogram of the exposome of serum sample
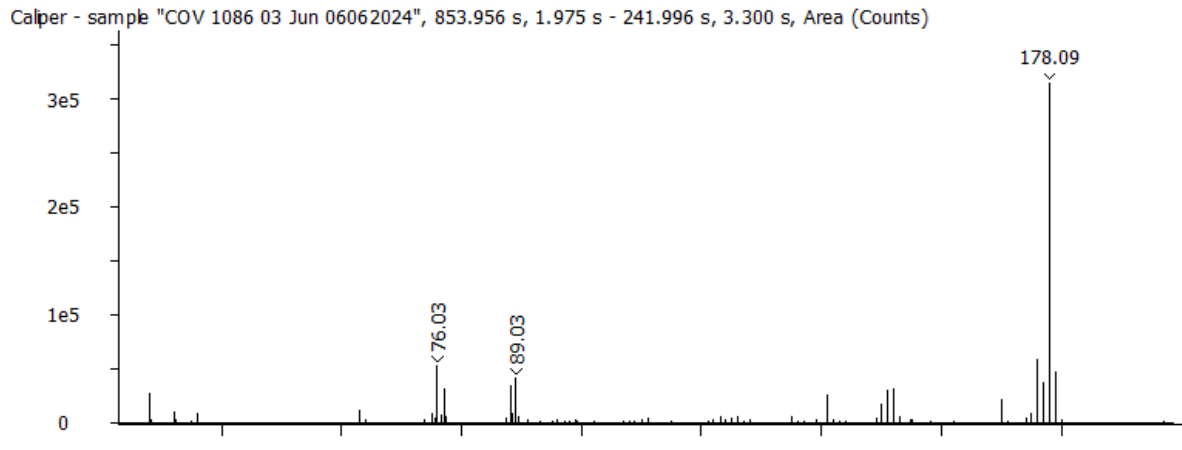
Figure 4.2: Mass spectra for anthracene.

Since many chemicals can be present in plastic and reagents, molecules were considered as exposome only if they were not present in the blank or their abundance was at least 10 times the abundance in the blanks. After the successful identification of molecules, 66 molecules are considered relevant for this study. Table (4.1) describes the identified molecules with their mass to charge and ratio and retention time in seconds.

Table 4.1: Identified molecules with their mass and retention time in seconds

Molecules	Mass (m/z)	RT (Sec)
Acenaphthylene	152	610
Acetophenone	105	304
Anthracene	178	854
Benzophenone	182	738
Benzyl alcohol	108	288
Biphenyl	154	550

Bis(2-ethylhexyl) phthalate (DEHP)	149	1470
Butylated Hydroxytoluene	205	650
Cetene (1-Hexadecene)	83	698
Dibutyl phthalate (DBP)	149	960
Diethyl Phthalate (DEP)	149	710
Dimethyl phthalate (DMP)	163	606
Pyrene	202	1106
Homosalate	138	926
Lilial	152	662
Naphthalene	128	378
p,p'-DDE	318	1146
Piperine	285	1758
p-Xylene	106	212
Fluoranthene	202	1066
Triacetin	103	506
Tributyl phosphate	99	742
Triethyl phosphate	155	326
Cotinine	98	798
Phenol, 2-(1,1-dimethylethyl)-5-methyl-	149	514
Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	340	1330
Phenol, 2,4-bis(1-methyl-1-phenyl ethyl)-	330	1434
Phenol, 2-nitro-	139	342
Phenol, 3-(1-methyl ethyl)-	121	402
Phenol, 3-methyl-	108	302
Phenol, 4-(1,1,3,3-tetramethyl butyl)-	135	758
Phenol, 4-(1,1-dimethyl propyl)-	135	554
Phenol, p-tert-butyl-	150	462
Phenylethyl Alcohol	92	326
Phthalic anhydride	104	494
Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	84	522
Tridecanal	82	642
1,2-Benzenedicarboxylic acid, bis(2-methyl propyl) ester	149	906
1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	261	1654
13-Docosenamide, (Z)-	72	1678
1-Butanamine, N-butyl-	86	254
1-Docosene	308	1138
1-Dodecanol	70	614
1-Hexadecanol	68	910
1-Hexanol, 2-ethyl-	57	278
1-Nonanol	98	346
1-Octadecanol	83	1060
1-Octadecene	97	846
1-Phenoxypropan-2-ol	94	422
Benzothiazole	135	414
Heptadecane	71	778
Mequinol	124	398

Naphtho[2,1-b]furan, dodecahydro-3a,6,6,9a-tetramethyl-	221	842
n-Hexyl salicylate	120	770
Nonanal	69, 98	316
Nonanoic acid	60	430
Octadecanamide	59	1690
Octadecane	85	850
Octanal, 2-(phenyl methylene)-	216	822
Octane, 1,1'-oxy bis-	84	752
o-Hydroxybiphenyl	170	656
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethyl pentyl ester	89	538
Propanoyl fluoride, 2,3,3,3-tetrafluoro-2-[1,1,2,3,3,3-hexafluoro-2-(heptafluoropropoxy)propoxy]- (PFASs)	169, 335	259
Tetradecanal	168	718
Tetradecane	85	554
Tributyl acetyl citrate	185	1190

4.1.2 Classification of recognised molecules

To understand the nature of each molecule, they were classified based on their characteristics into 9 categories. Figure 4.2 illustrates the distribution of nine distinct categories of molecules within the exposome. It is seen that each category including household chemicals, industrial chemicals, and pesticides, all account for twenty percent implying a uniform dispersal of chemicals across these nine types. The next step is Personal Care Products (PCPs) which take up around fifteen percent which indicates the importance attached to them. Polycyclic Aromatic Hydrocarbons (PAHs) make up approximately one-tenth (8%) of the entire content. Moreover, Phthalates (6%), Food Additives (4%), Organophosphorus Compounds (3%), and Other Exosomes (4%) were found in the analysis.
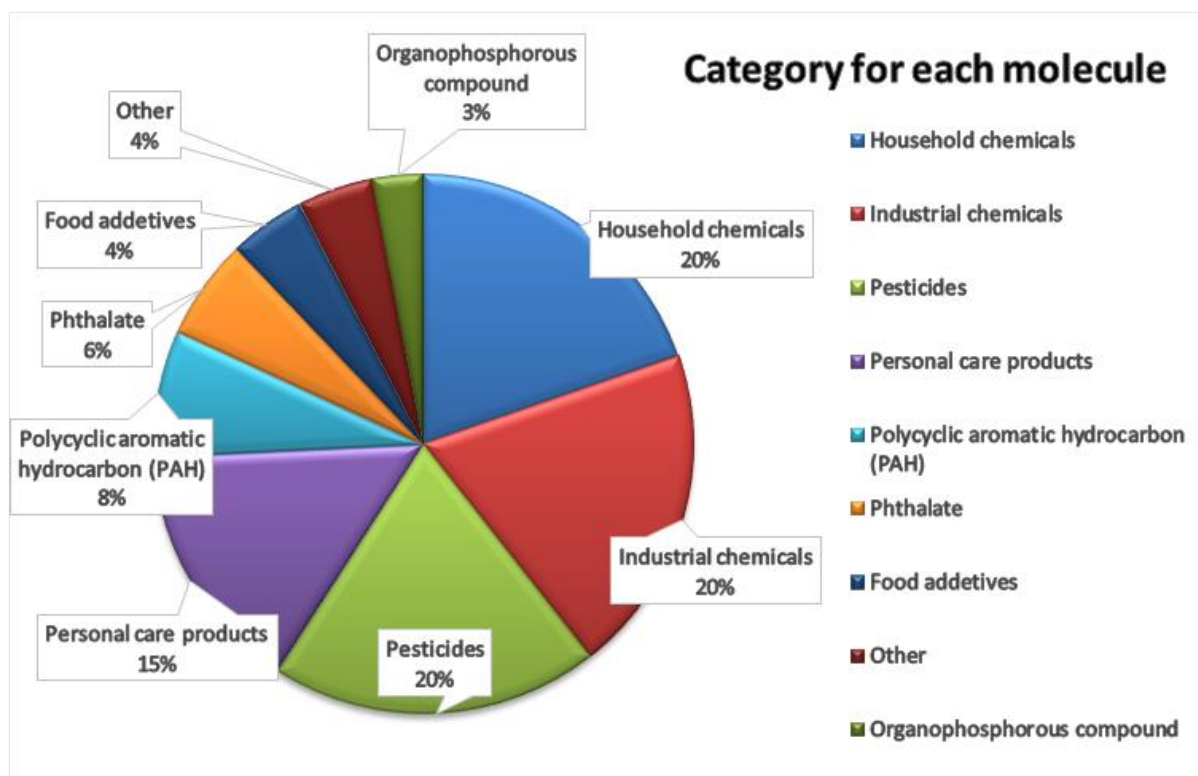
Figure 4.3: Graphical representation of exposome within each category

For understanding the behaviour of the 9 categories, the molecules in each category are plotted with respect to the mass and retention time. Figure 4.3 to Figure 4.11 depicts the mass vs retention time plot for each category representing the scattering of each molecules within a particular category.
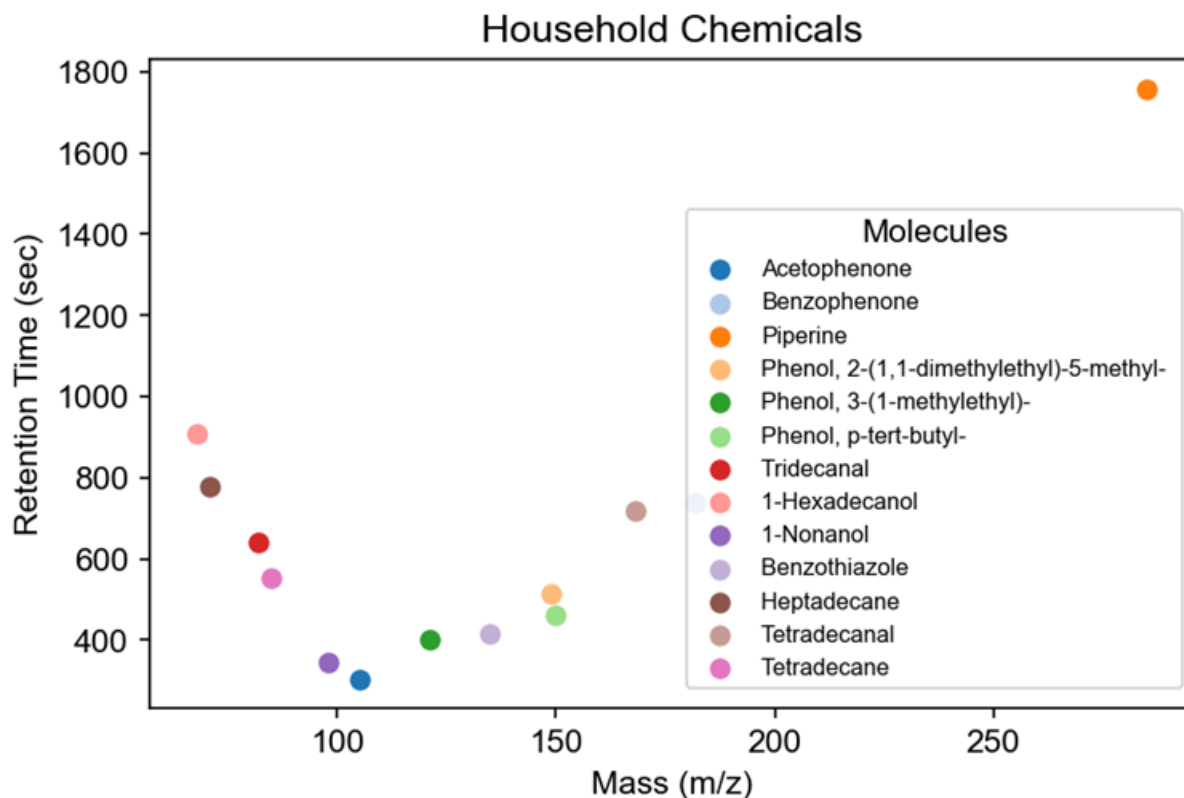
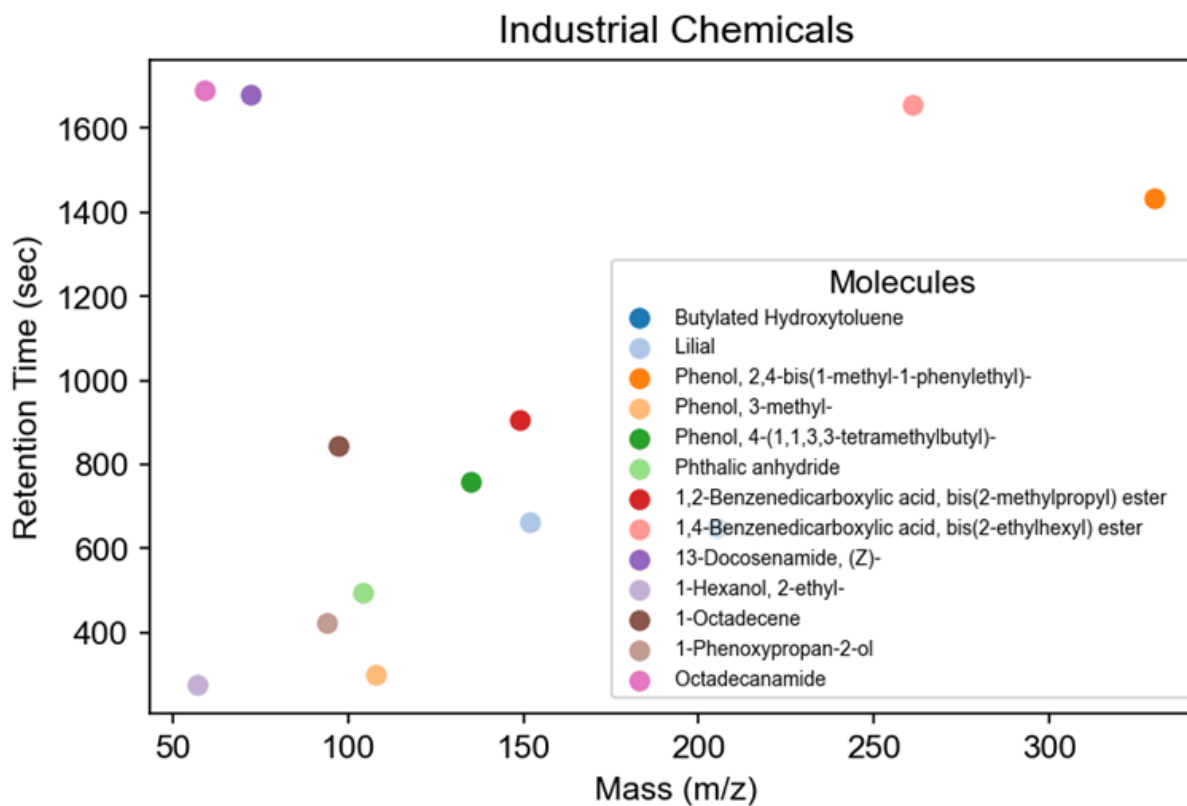**Figure 4.4: Exposome from Household Chemicals**
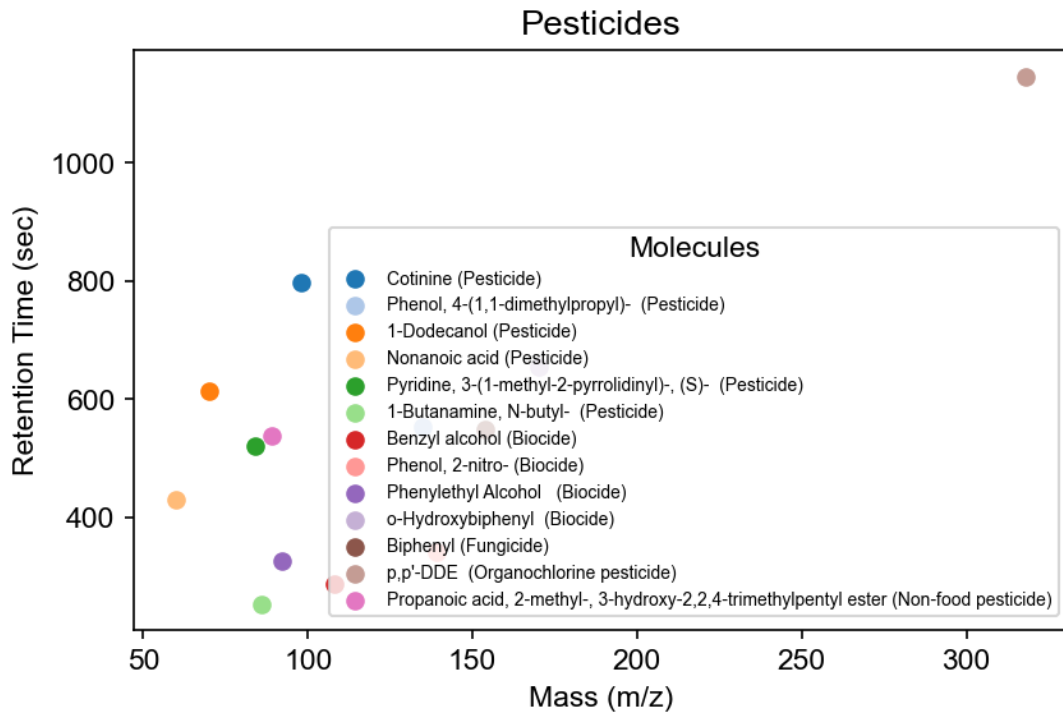

Figure 4.5: Exposome from Industrial Chemicals

Pesticides

Figure 4.6: Exposome from pesticides

Legend (Molecules):
- Cotinine (Pesticide)
- Phenol, 4-(1,1-dimethylpropyl)- (Pesticide)
- 1-Dodecanol (Pesticide)
- Nonanoic acid (Pesticide)
- Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (Pesticide)
- 1-Butanamine, N-butyl- (Pesticide)
- Benzyl alcohol (Biocide)
- Phenol, 2-nitro- (Biocide)
- Phenylethyl Alcohol (Biocide)
- o-Hydroxybiphenyl (Biocide)
- Biphenyl (Fungicide)
- p,p'-DDE (Organochlorine pesticide)
- Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester (Non-food pesticide)

Personal Care Products

Figure 4.7: Exposome from PCPs

Legend (Molecules):
- Cetene (1-Hexadecene)
- Homosalate
- Triacetin
- Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-
- 1-Docosene
- 1-Octadecanol
- Octadecane
- Octanal, 2-(phenylmethylene)-
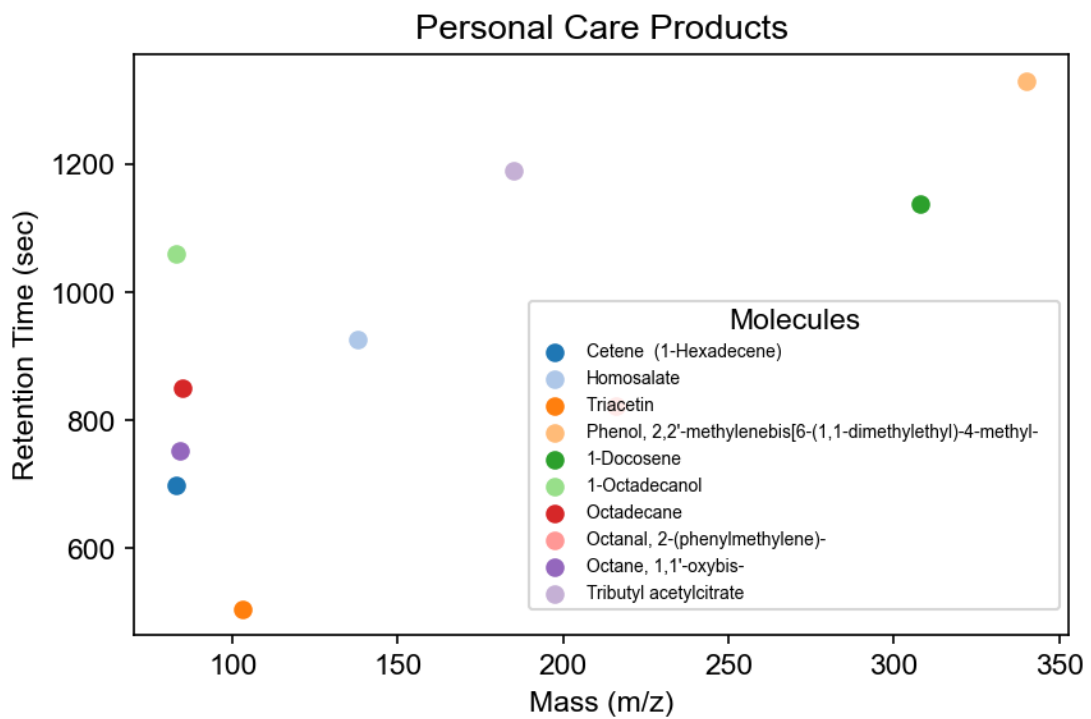- Octane, 1,1'-oxybis-
- Tributyl acetylcitrate
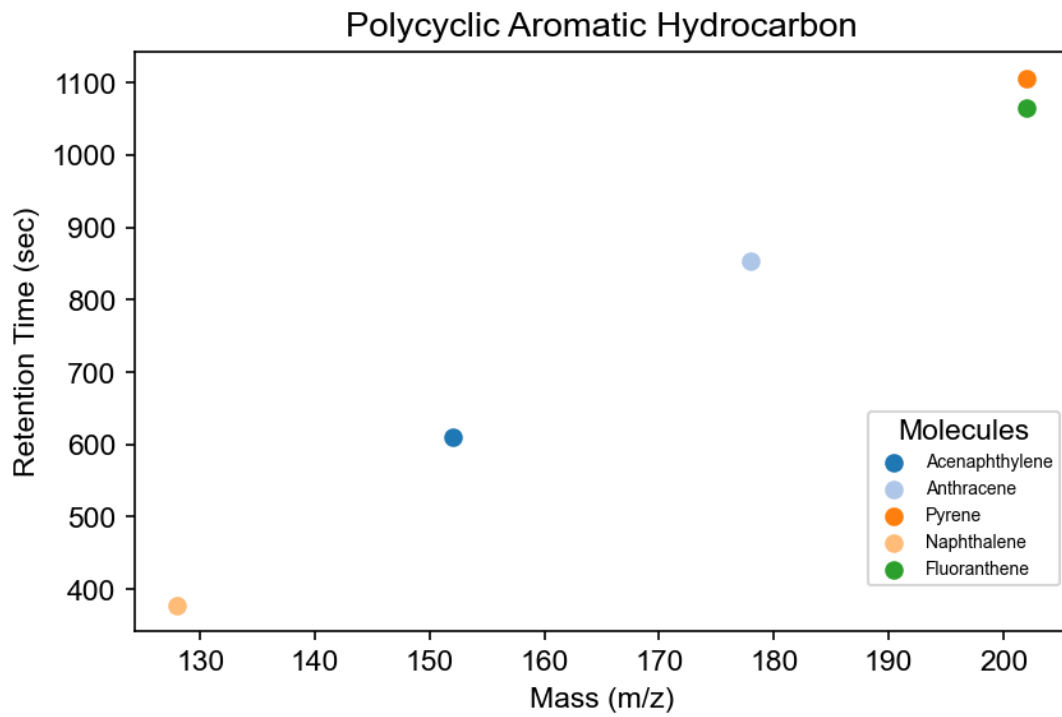
Figure 4.8: Exposome from PAH
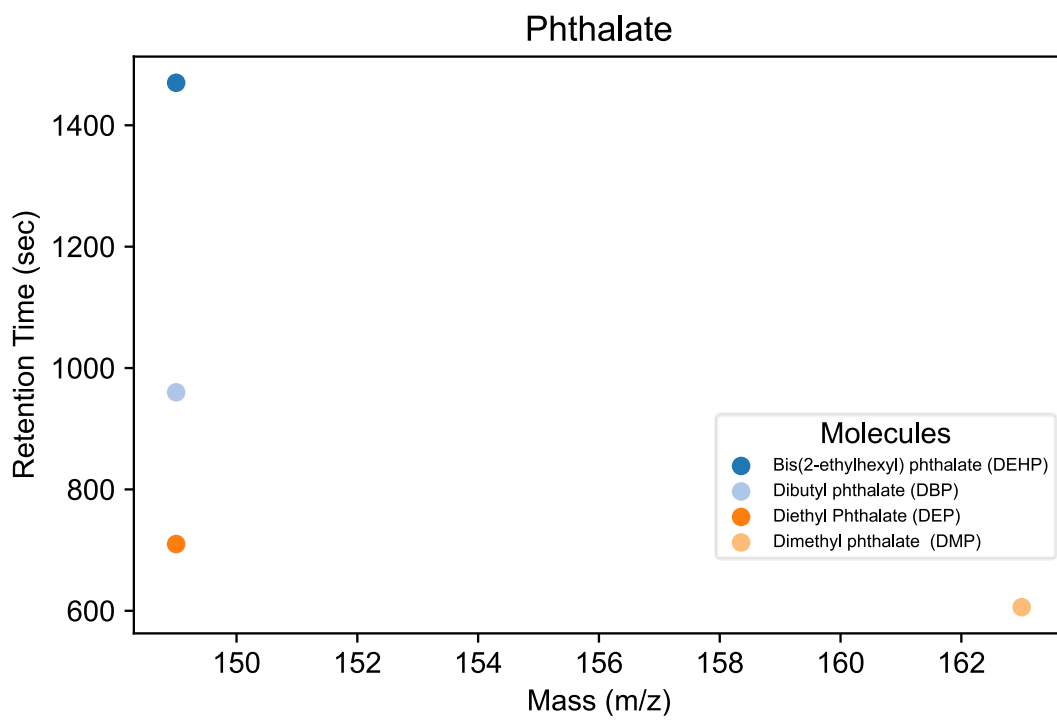
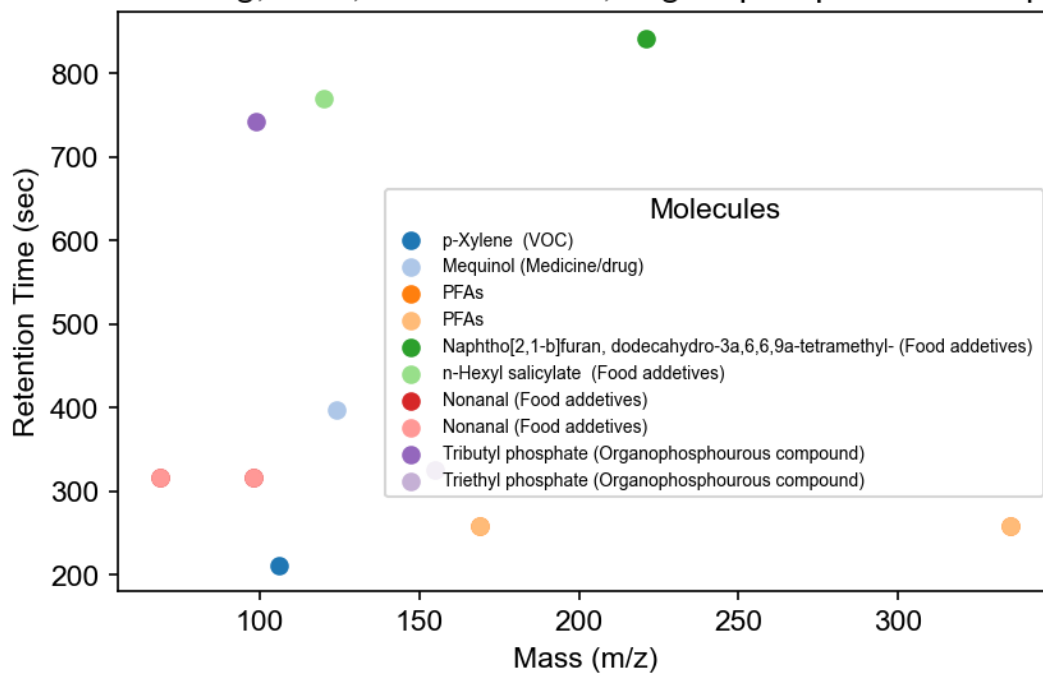**Figure 4.9: Exposome from phthalate**

Figure 4.10: Exposome from Medicine/drugs, VOC, Food Additives, Organophosphorus Compounds

4.1.3 Molecules Distribution

The identified and categorized molecules were further distributed amongst two variables i.e. sex and age groups. This helps to understand how the exposome is distributed in females and males and their different age groups i.e., Young (25-35 yrs.), Middle-aged (35-50 yrs.), Old (51-71 yrs.).

Heat maps were employed to provide a more accurate representation of exposome molecules across our samples, including sex and age groups of young, middle-aged, and old group individuals.

Figure 4.11: Heatmap showing the distribution of exposome in the analysed cohort. A red box indicates that the molecule was identified while the white box indicates that the molecule was not present. Samples are clustered based on sex.
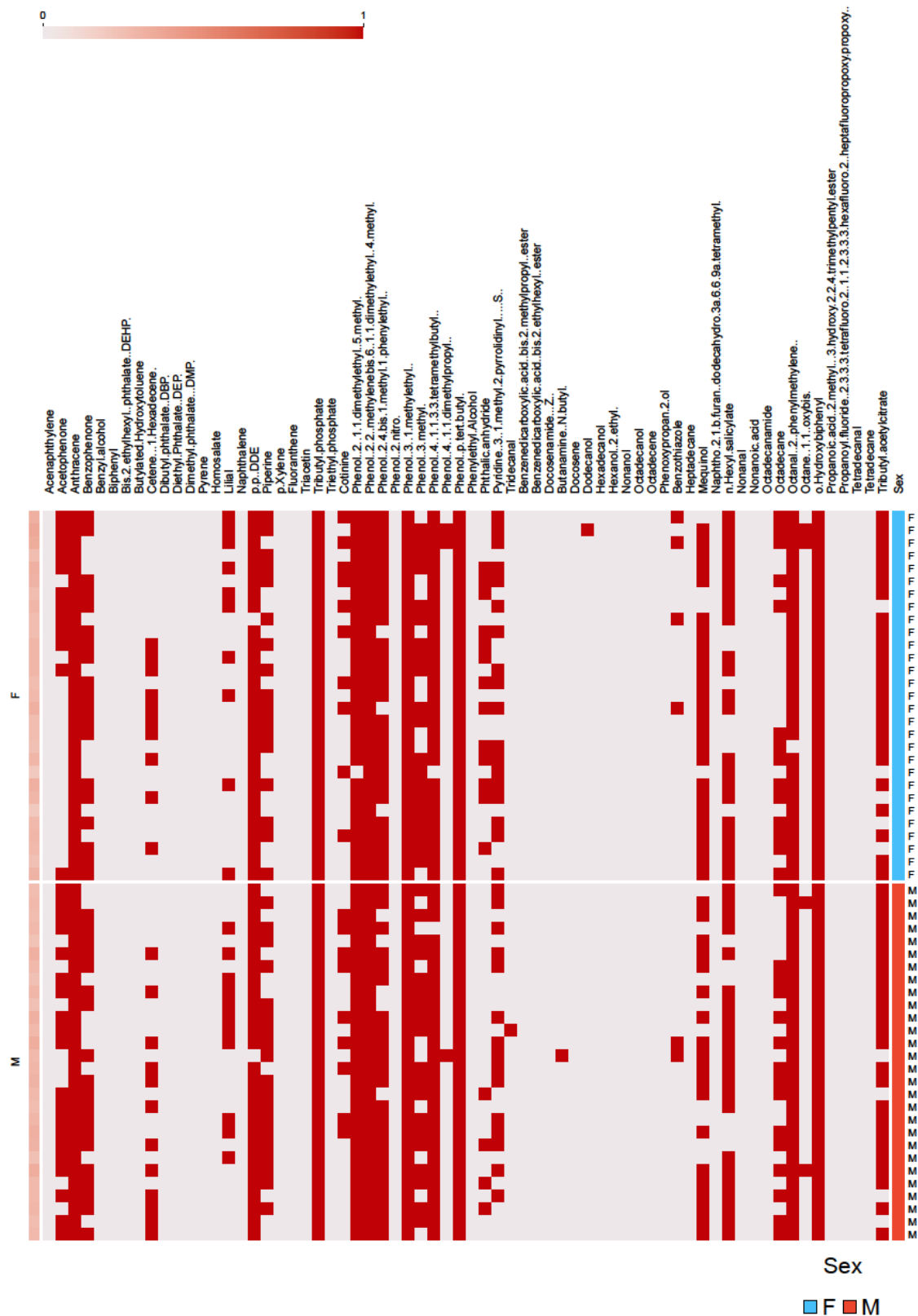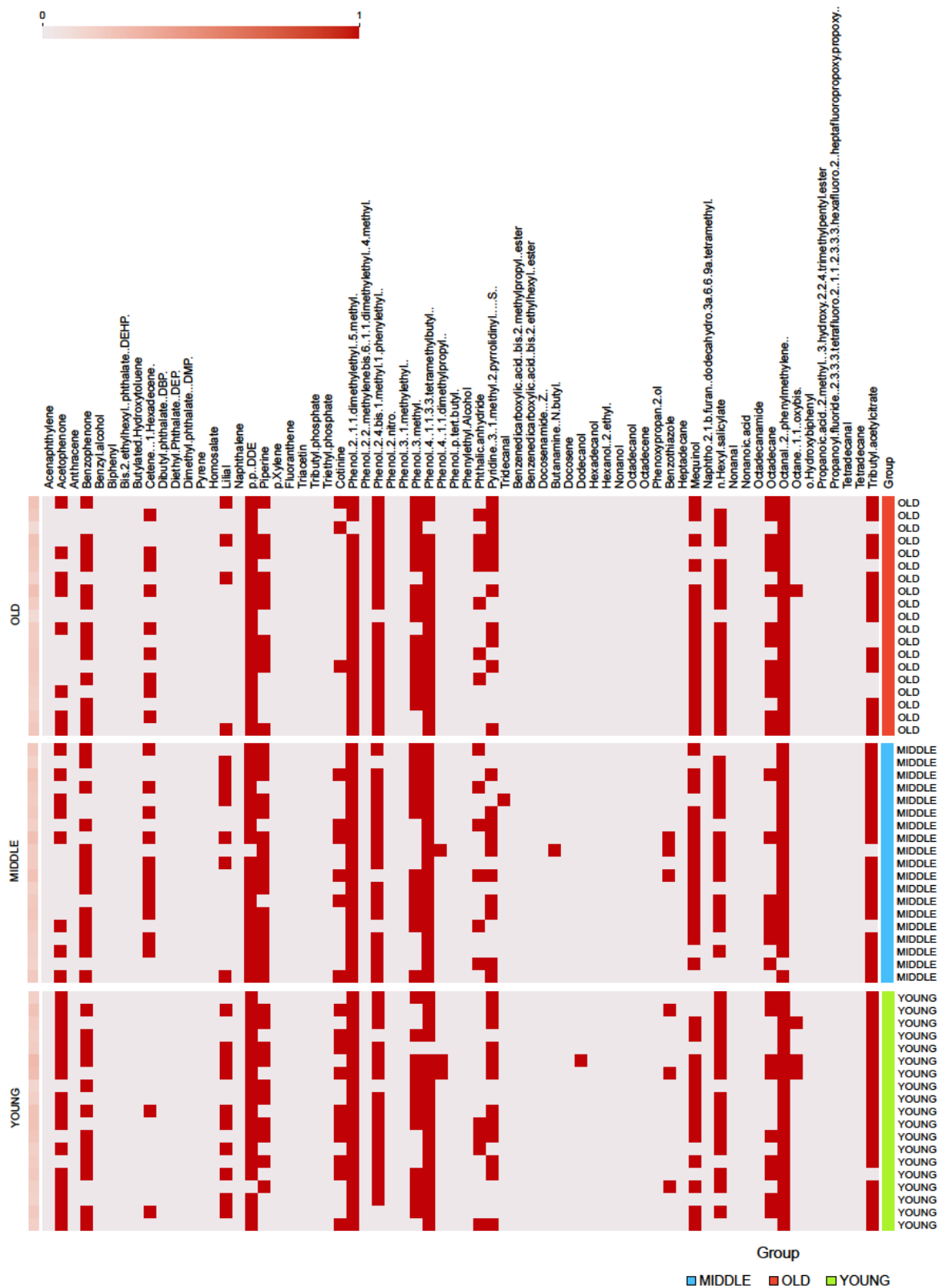
Figure 4.12: Heatmap showing the distribution of exposome in the analyzed cohort. A red box indicates that the molecule was identified while the white box indicates that the molecule was not present. Samples are clustered based on age.

➢ **Sex**

In figure 4.13 reports the distribution of exposome on the overall population based on female and male, respectively.
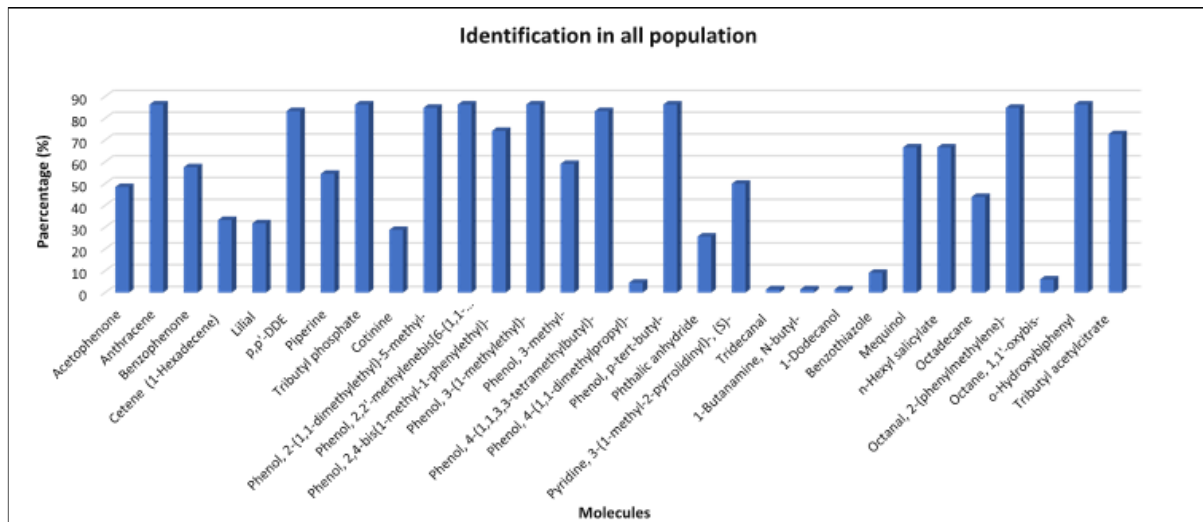

Figure 4.13: Distribution of exposome among all population

Figure 4.13 highlights the relative abundance of different chemical groups. It can be seen that the identification amongst all populations constitutes more than 60% exposure for molecules in the household, industrial chemicals, and the PAH category, some exposomes from the pesticide category were detected in 80% whereas the molecules from the remaining categories are observed in less than 20%.

Figure 4.14: Distribution of exposome among the female population

Figure 4.14 depicts the distribution of various chemical categories within the exposome of the female population, showcasing the differential exposure to various compounds. More than 35% of molecules from household and industrial chemical categories were found in the female population. There was a major observation of anthracene exposure among females, with more than 40% being noted.
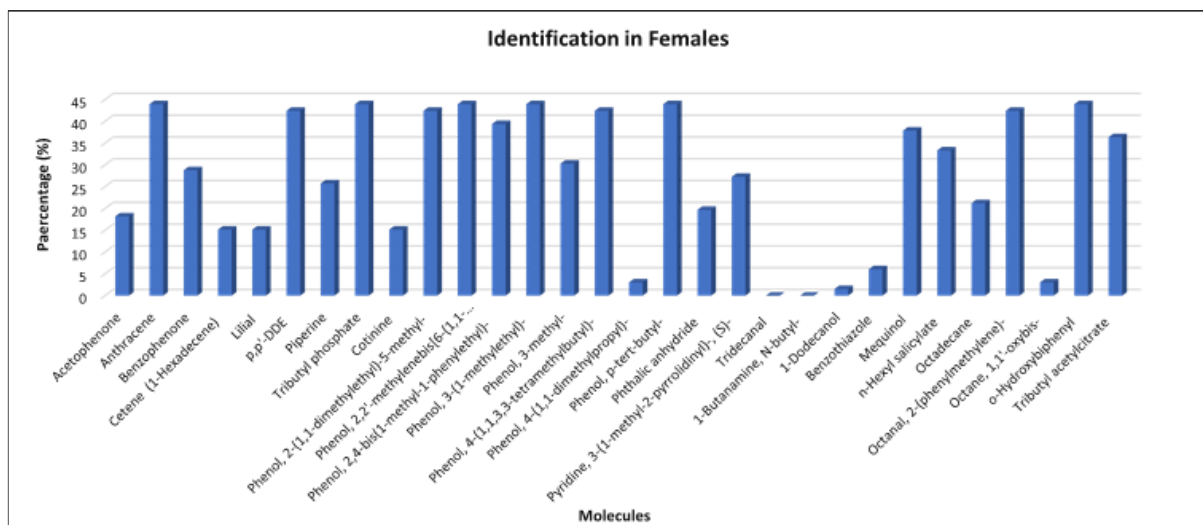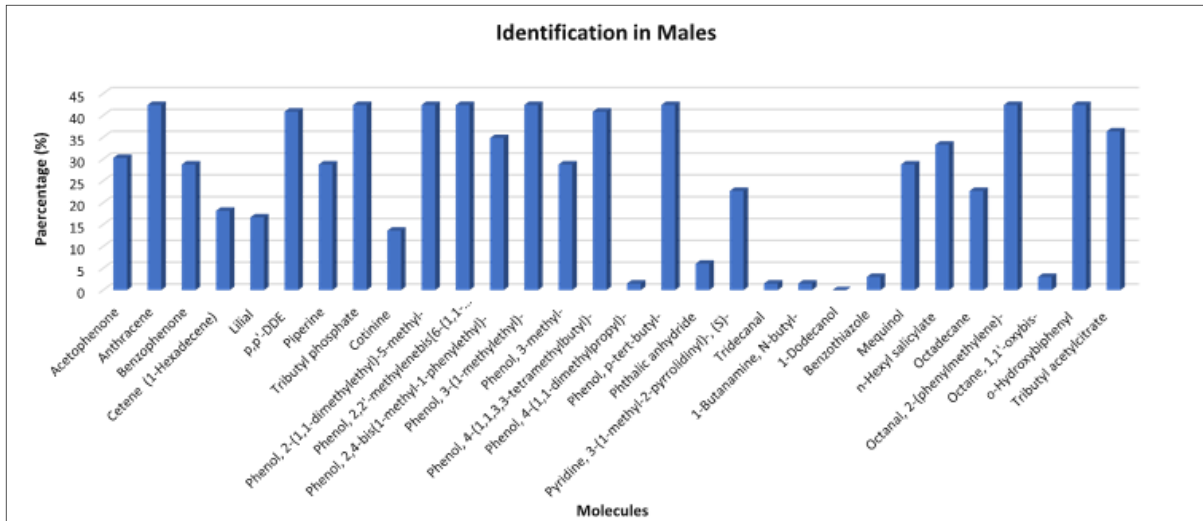
Figure 4.15: Distribution of exposome among the male population

Figure 4.15 illustrates the distribution of various chemical categories within the exposome of the male population. The analysis showed that less than 5% of molecules from pesticides and tridecanal from household chemicals were detected. However, more than 40% of molecules from industrial chemicals, PCPs, as well as PAH and organophosphorus molecules were observed.
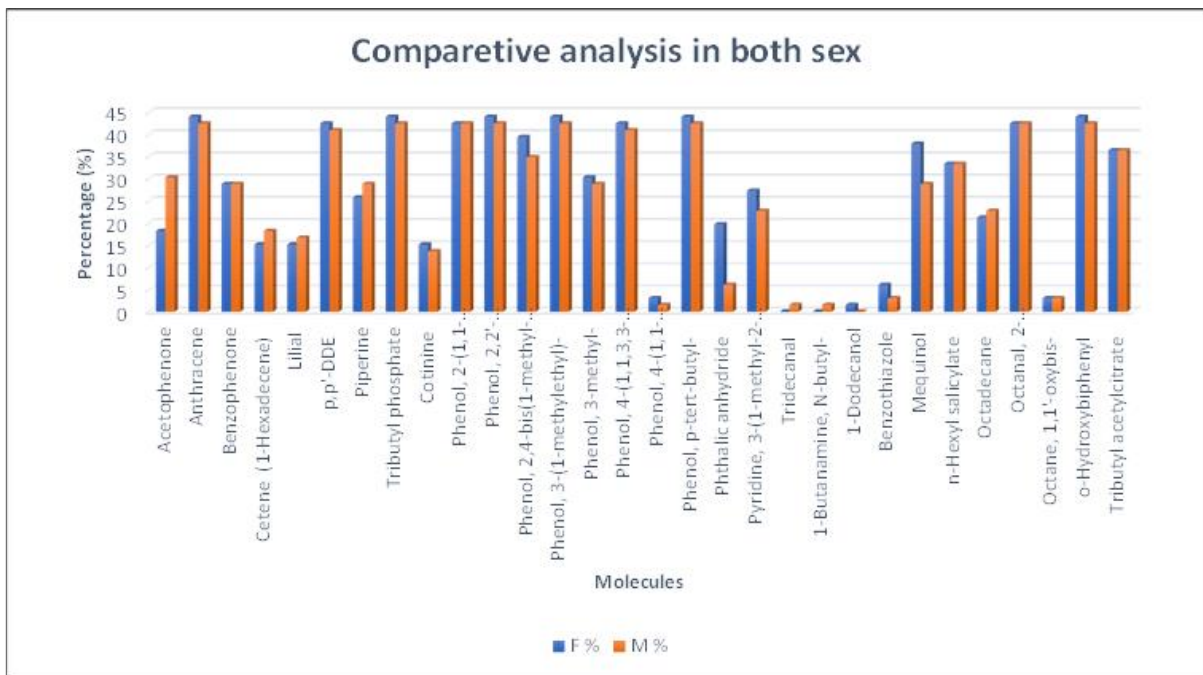
Figure 4.16: Comparative analysis of exposome among both sex

In Figure 4.16, the differences in exposome percentages between male and female populations are illustrated, providing insight into the gender-specific impacts of various chemical exposures. More than 40% of molecules from all categories were found to be exposed in both females and males, except the mequinol molecule from the "other" category. The majority of females showed higher levels of exposure compared to males. However, some molecules were found to be exposed only 5% in males and were not detected in the female population. Anthracene exposure was observed to be greater in females than in males.

- ➢ **Age Groups**

As per the design of the study, a thorough analysis is performed on different age groups ranging from young to old. The age groups are divided as follows:

Young (20-35 yrs.)

Figure 4.17: Distribution of exposome among young age group (20-35yrs)

The percentages of each identified exposome molecule in the young age group are illustrated in Figure 4.17. Certain molecules, such as tridecanal from household chemicals, and 1-Butylamine, N-butyl from pesticides, were not observed in the young age group. Meanwhile, some were observed in less than 5% of cases, and the majority showed around 29% exposure to molecules from households, industrial chemicals, and PCPs.

Middle (20-35 yrs.)

Figure 4.18: Distribution of exposome among middle age group (36-50yrs)

The percentages of each identified exposome molecule in the middle age group are depicted in Figure 4.18. Less than 5% of exposure was observed for some molecules in the household, pesticide, and PCP categories. Furthermore, 1-Dodecanol was not detected in the middle age group. It was observed that 30% of exposure for the exposome was from each category.
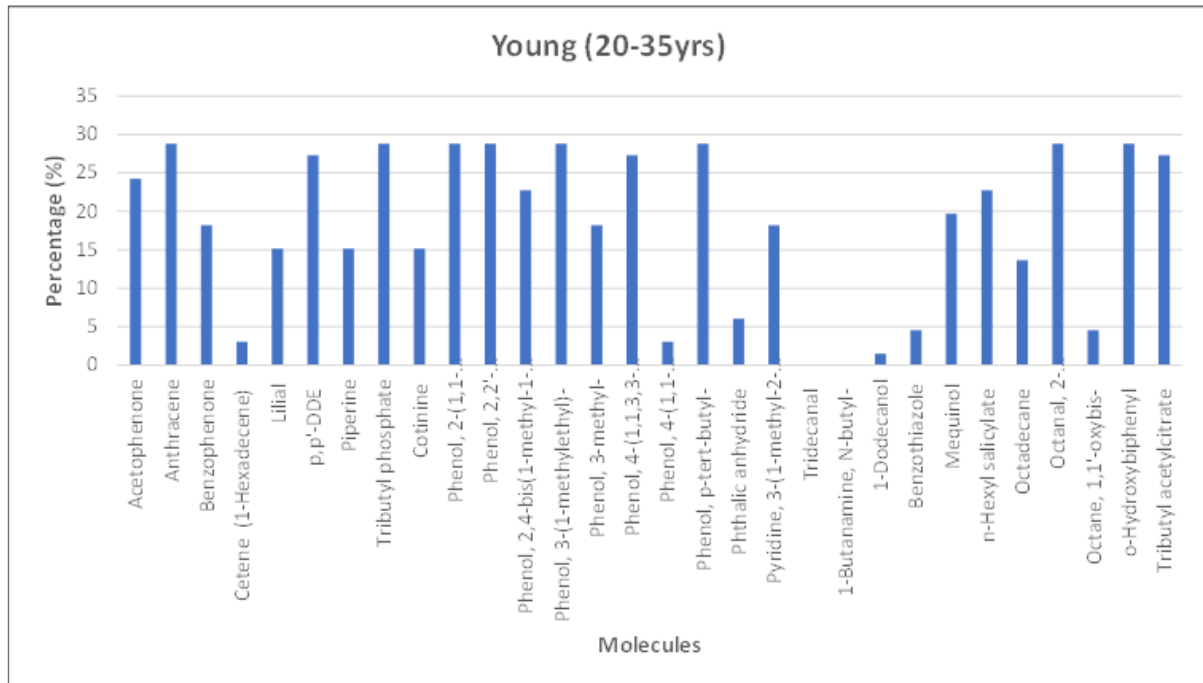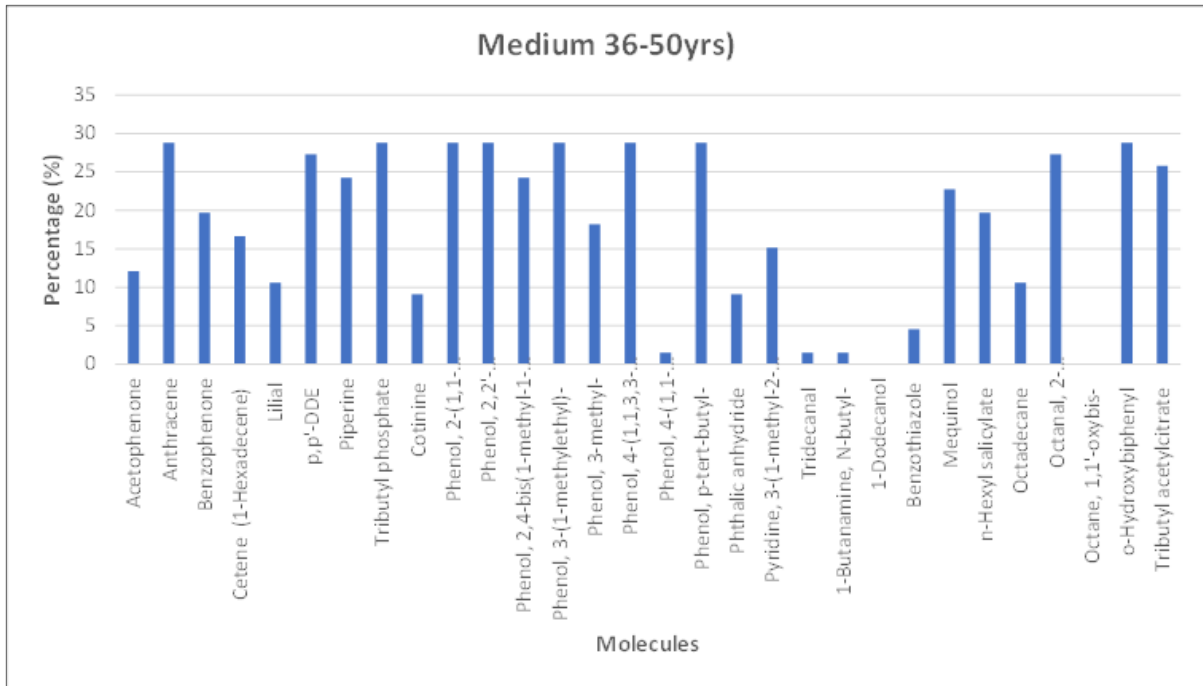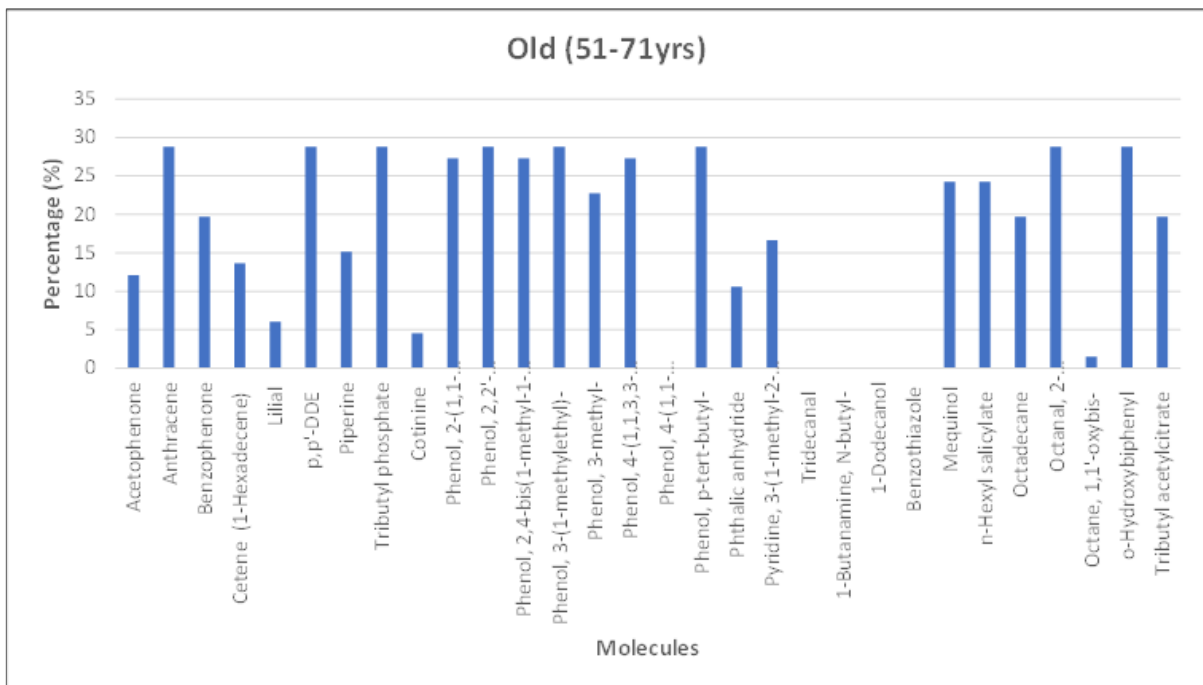
Old (51-70 yrs.)

Figure 4.19: Distribution of exposome among old age group (51-71yrs)

The percentages of each identified exposome molecule in the old group are depicted in Figure 4.19 Some molecules are not detected in the old age group across all categories. On the other

hand, more than 25% of exposure to household and industrial chemicals, organochlorine pesticides, and some PCPs in most of the population.

Subsequently, all three groups were analysed together to understand how exposomes are differently behaved compared to age criteria.
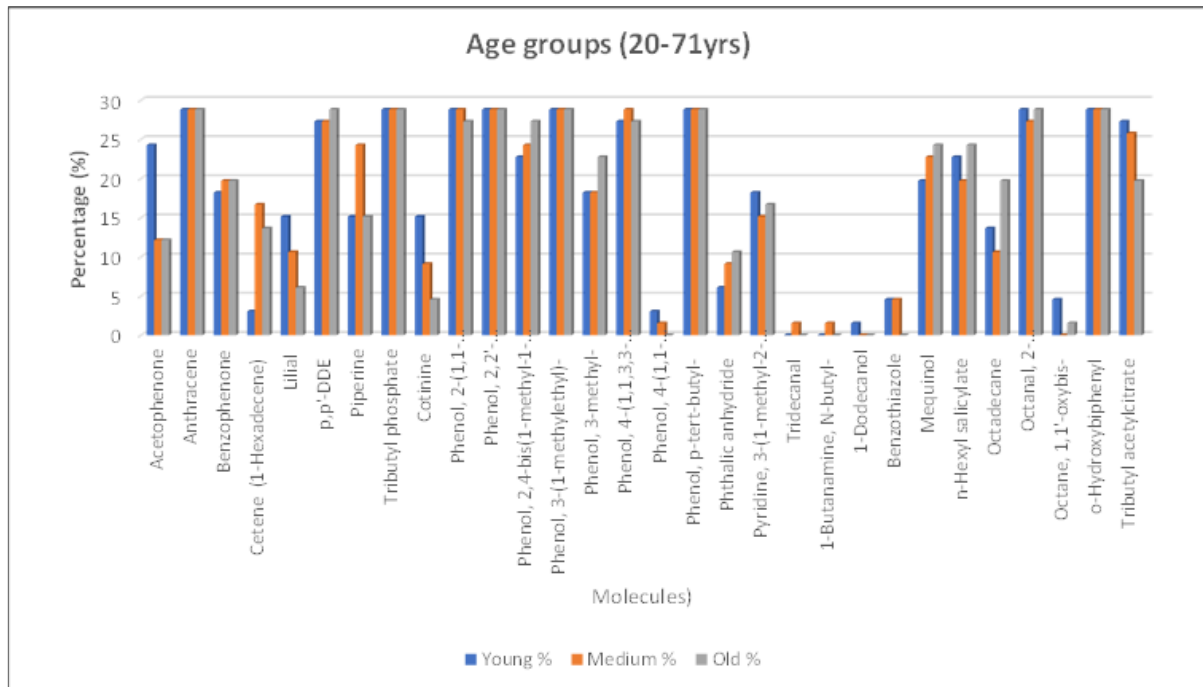
Figure 4.20: Comparative analysis of exposome among all age groups

The percentages of each identified exposome molecule reveal that more than 25% of exposures occurred among all groups for all categories. Molecules were not in the old age group compared to the other two. The study found around 30% similarity in exposure across all age groups, while less than 5% of exposure to industrial chemicals and PCPs was detected in all age groups. Younger age group showed slightly higher exposure. High exposure to the p,p'-DDE organochlorine pesticide was identified in the older age group. Tridecanal and 1-Butanamine, N-butyl were only found in the middle age group.

4.1.4 Comparison of Category Distribution of Exposomes

We then investigated how each molecule from the respective category impacts on age and sex.

➢ Household Chemicals

Figure 4.21 helps to observe the distribution of each household chemical molecule across males and females to understand the difference in exposure based on sex, considering the

percentage. Figure 4.22 and Figure 4.23 depicts each age group within each sex to see if the impact of exposure varies across different age groups as per the study design.
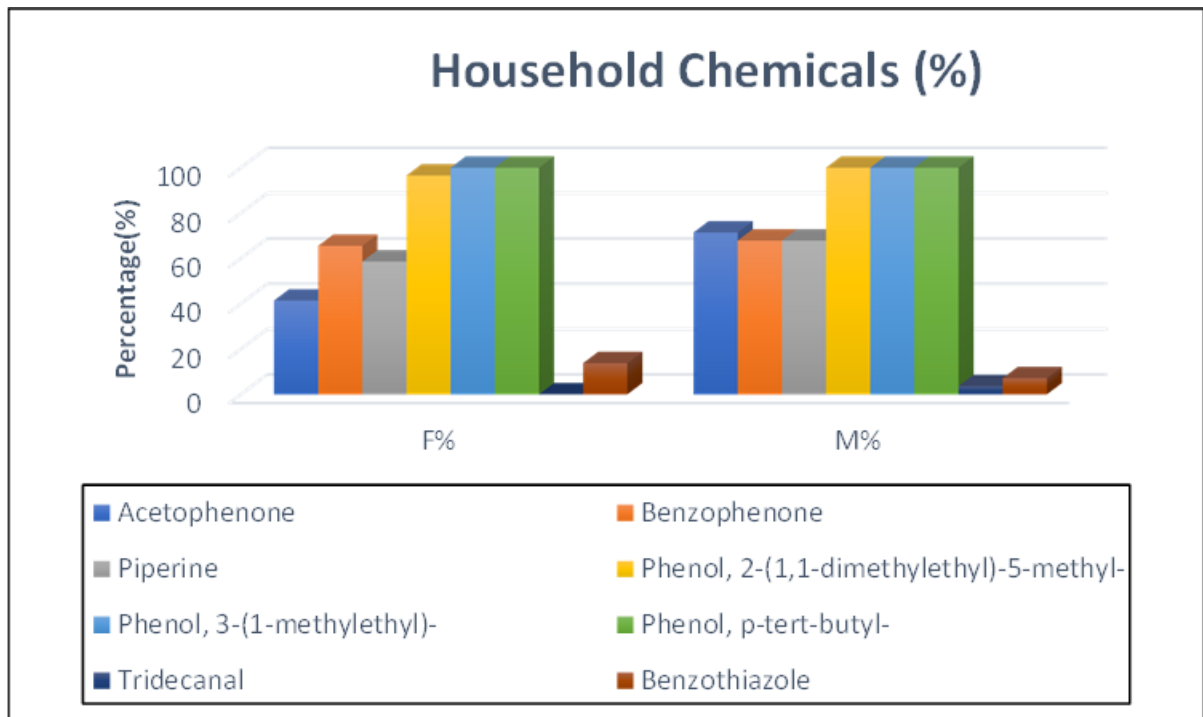
Figure 4.21: Comparison for distribution of household chemicals between both sexes

The household category molecule percentages in both sexes are shown in Figure 4.21. No significant differences are present. Benzothiazole exposure is over 10% in females compared to males, while acetophenone exposure is over 60% in males and less than 40% in females.
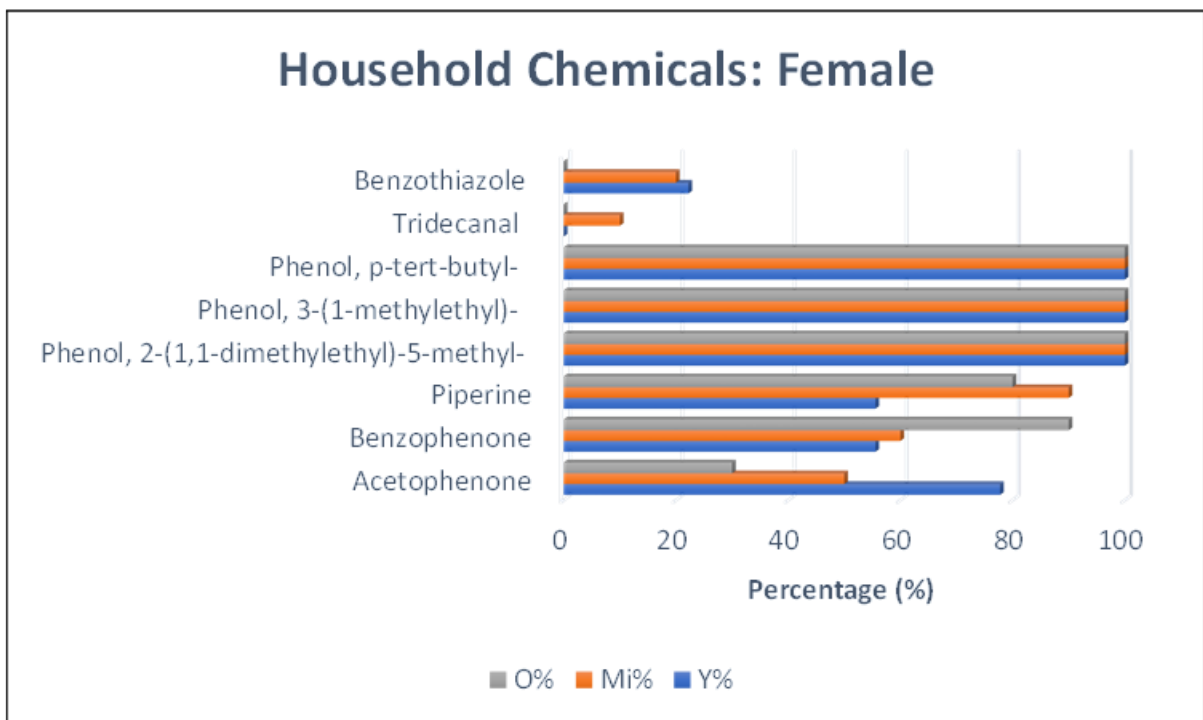
Figure 4.22:Household chemicals distribution among all age groups among females

Figure 4.22 illustrates the presence of each household molecule in females across three distinct age groups. Tridecanal exposure is detected in 15% of individuals in the middle age group, but it was not found in the other two groups. Acetophenone exposure exceeds 65% in the young age group. The older age group shows approximately 85% exposure to benzophenone, while the remaining groups exhibit less than 60% exposure.
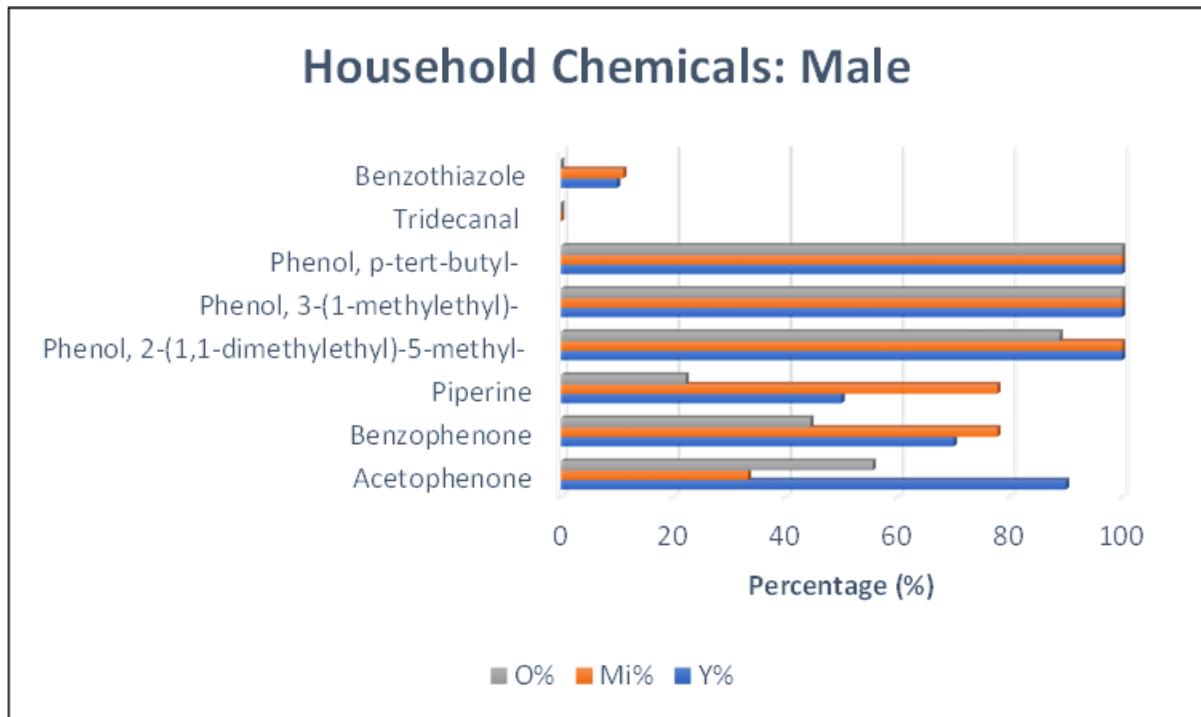
Figure 4.23: Household chemicals distribution among all age groups among males

In Figure 4.23, each household chemical molecule is depicted as a percentage. Tridecanal does not appear in any of the three age groups. Acetophenone exposure is higher in the young age group at 85%, compared to 55% in the old group and less than 40% in the middle age group. The old age group does not have exposure to benzothiazole.

➢ Industrial Chemicals
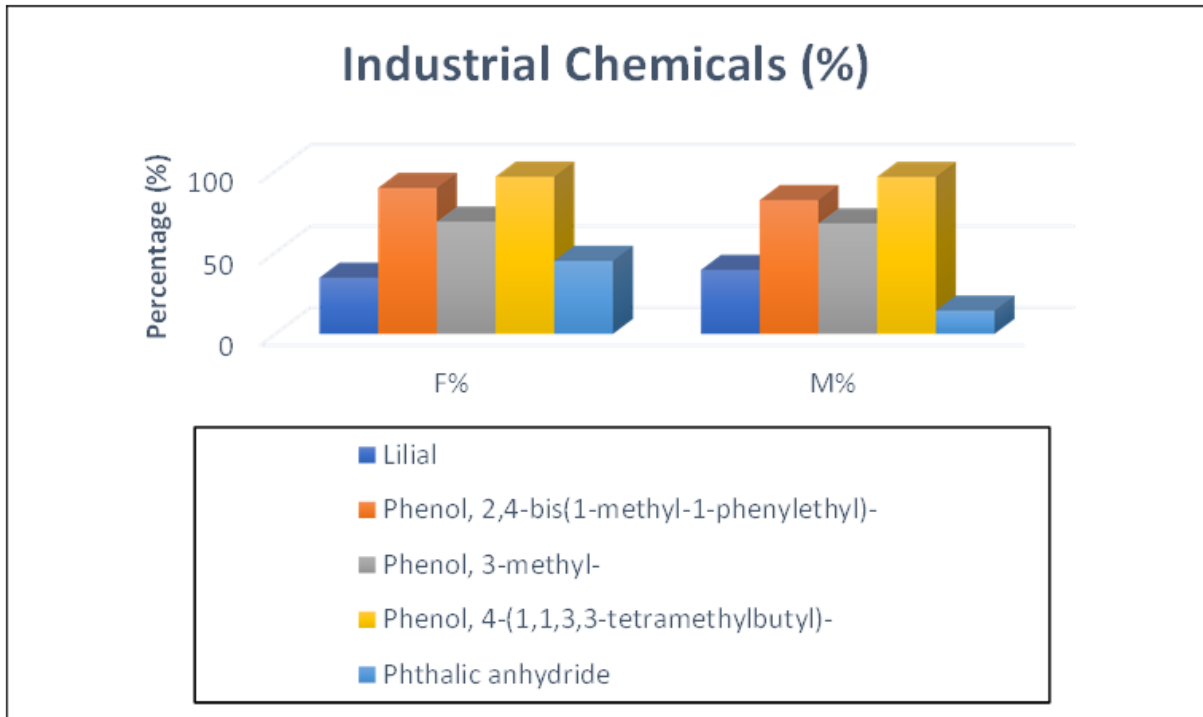
Figure 4.24:Industrial chemicals distribution among all age groups among females

As shown in Figure 4.24 little exposome difference is present between males and females. The only notable finding is that phthalic anhydride exposure is around 40% in females but only 10% in males.
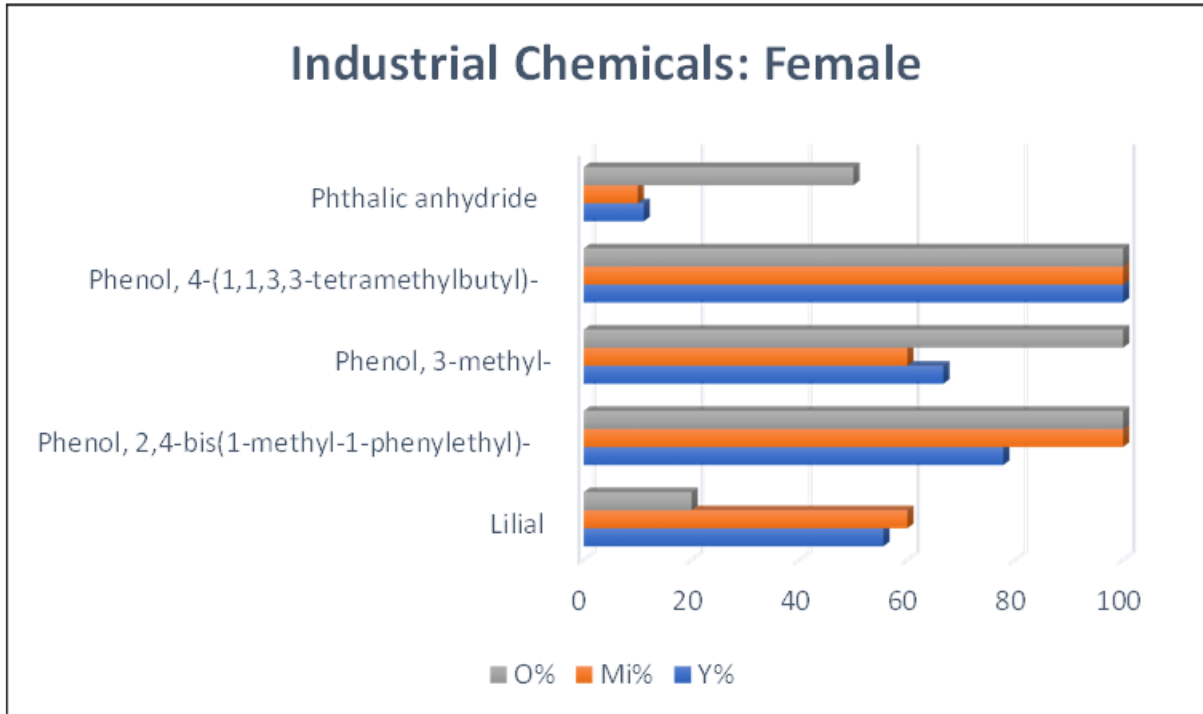
Figure 4.25: Industrial chemicals distribution among all age groups among females

The information presented in Figure 4.25 displays the presence of industrial chemicals within females in different percentages among age groups. When examining the specific female population, it is evident that more than 50% of the older age group were exposed to phthalic

anhydride, whereas less than 15% of the young and old age groups were exposed. Among all the molecules, the older age group had more than 50% exposure, except for lilial, which had less than 20% exposure.
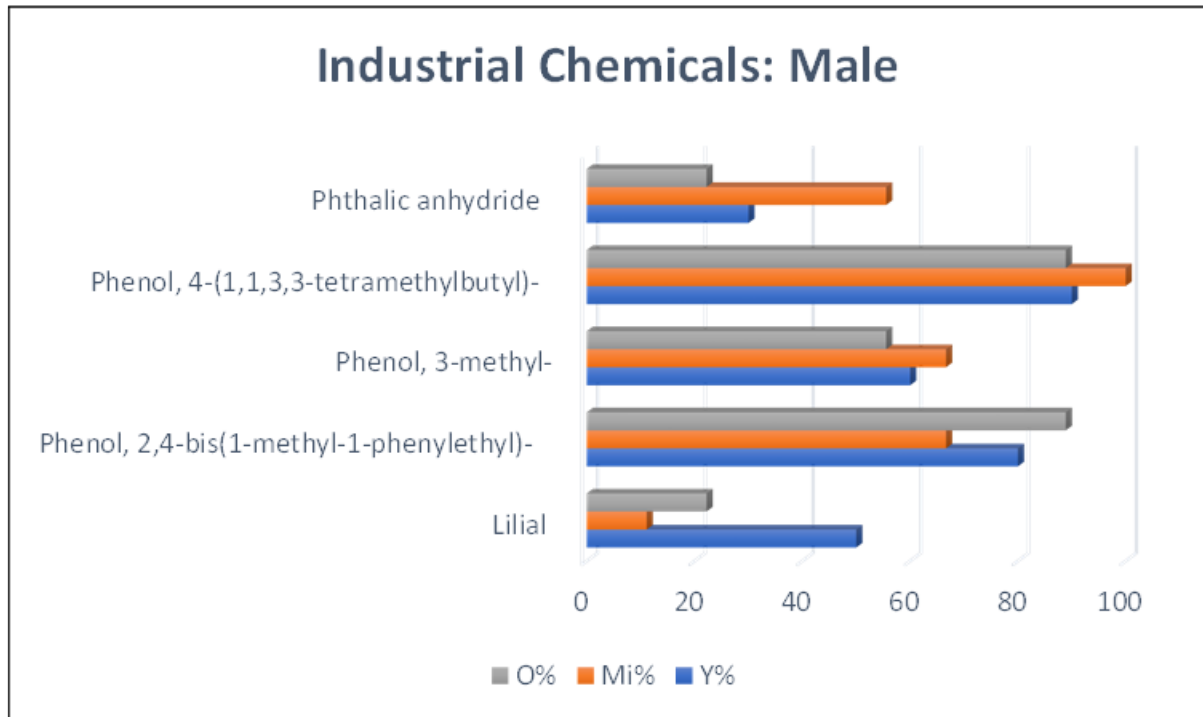
Figure 4.26: Industrial chemicals distribution among all age groups among males

In Figure 4.26 is reported the proportion of industrial chemical molecules present in the male population. The middle age group shows over 90% exposure to phenol, 4-(1,1,3,4-tetramethylbutyl)- in comparison to all the other molecules. There is a noticeable contrast in exposure to phthalic anhydride, with 55% in the middle age group, but less than 35% in both the young and old age groups. The exposure to Lilial is only 15% in the middle age group, which is the lowest among all the molecules.

➢ Pesticides
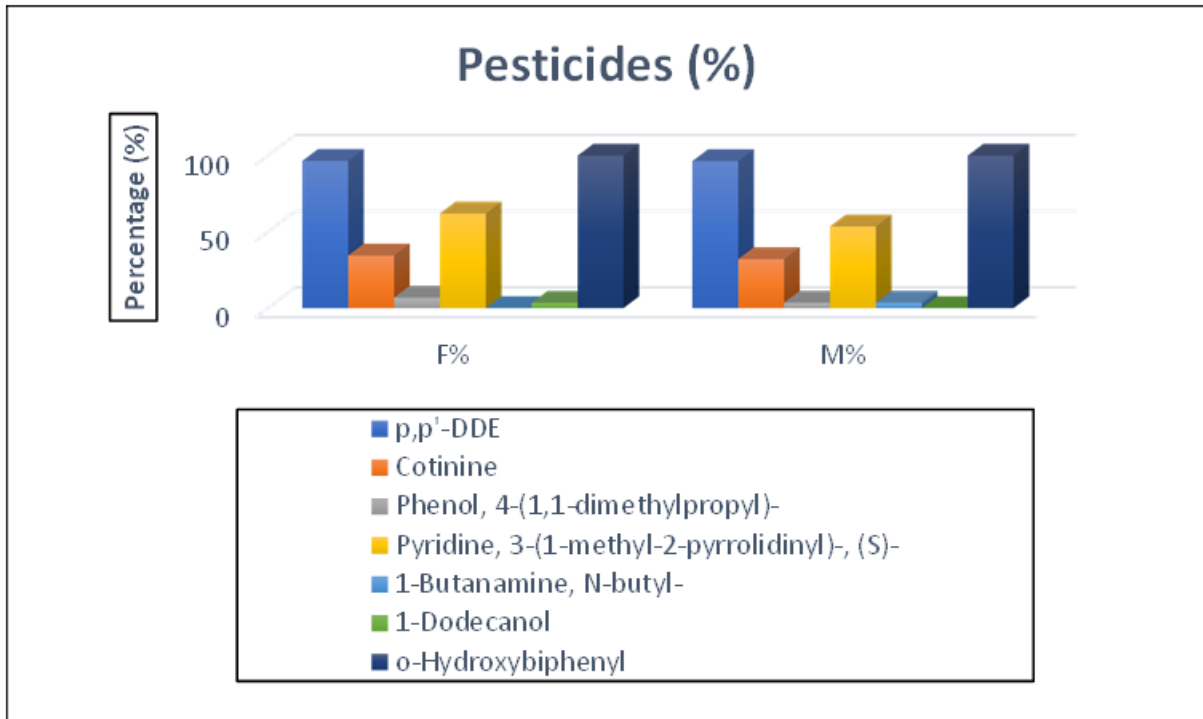
Figure 4.27: Comparison for distribution of pesticides between both sexes

The information in Figure 4.27 shows the data for molecules belonging to the pesticides category in both females and males. No significant differences were detected across genders, although a slight variation is noticeable for phenol, 4-(1,1-dimethylpropyl)- and pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- in females compared to men.
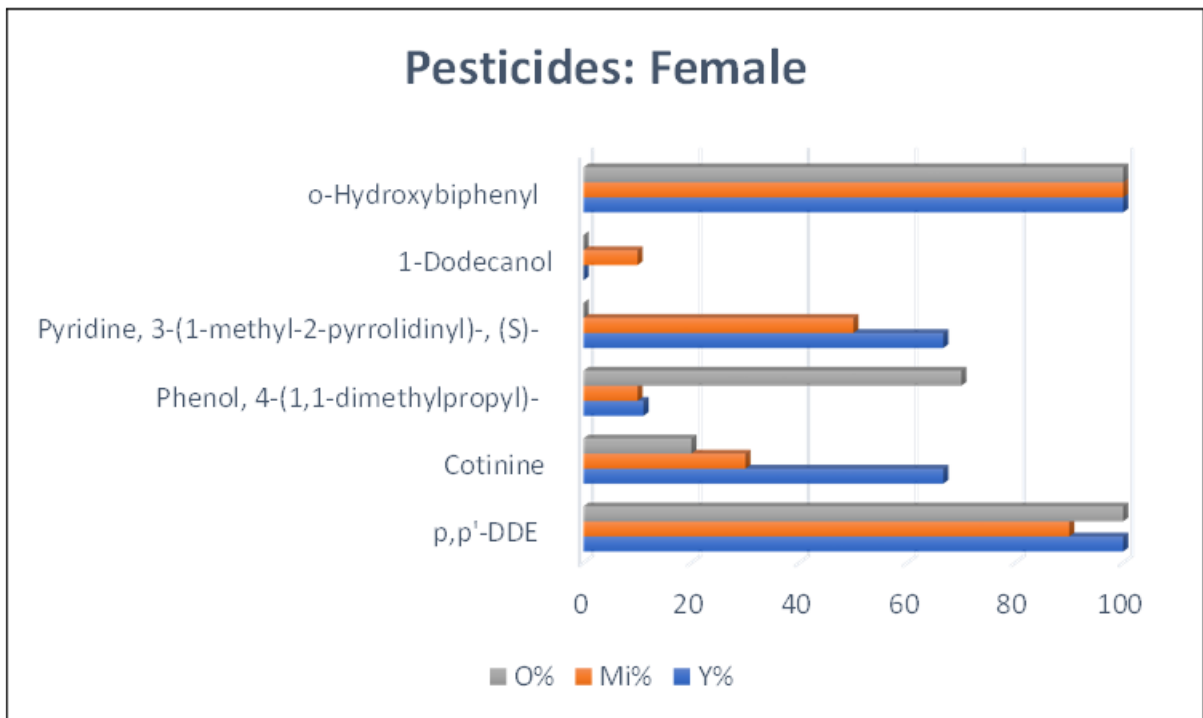
Figure 4.28: Pesticides distribution among all age groups among females

In Figure 4.28, the data illustrates the exposure to pesticides among females in all three age groups. All femles across all age groups showed 100% exposure to o-hydroxybiphenyl. The

middle age group was the only segment observed to have less than 15% exposure to 1-dodecanol, and exposure was not identified for the other two groups. More than 60% of the old age group was exposed to phenol, 4-(1,1-dimethylpropyl), while less than 20% of the exposure was observed in the other two age groups. There was a higher exposure to cotinine in the young age group, with less than 20% exposure observed in the old age group.
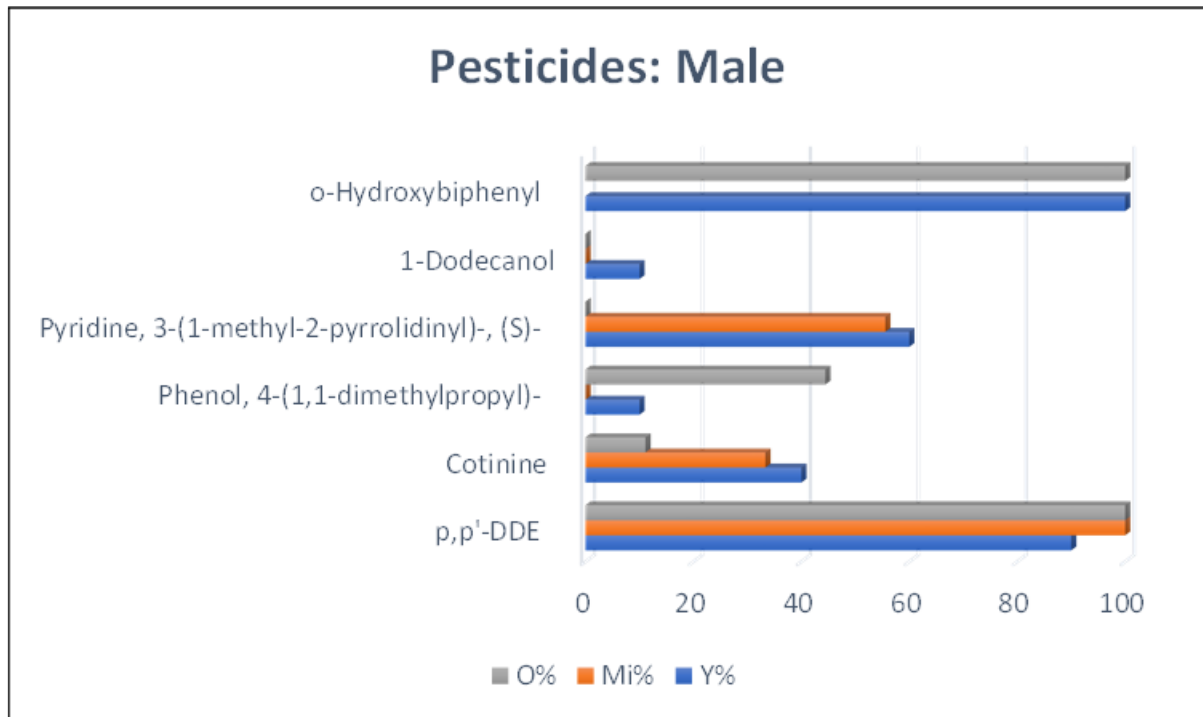
Figure 4.29: Pesticides distribution among all age groups among males

Figure 4.29 shows how pesticides are distributed among males across three different age groups. In the middle-aged group, exposure to pesticides is minimal, while 100% exposure to organochlorine pesticides is observed. Among all the molecules, 1-dodecanol has the lowest exposure, being less than 5% in both the middle and old age groups. O-Hydroxybiphenyl is not exposed to people in their middle age.

Personal care products (PCPs)
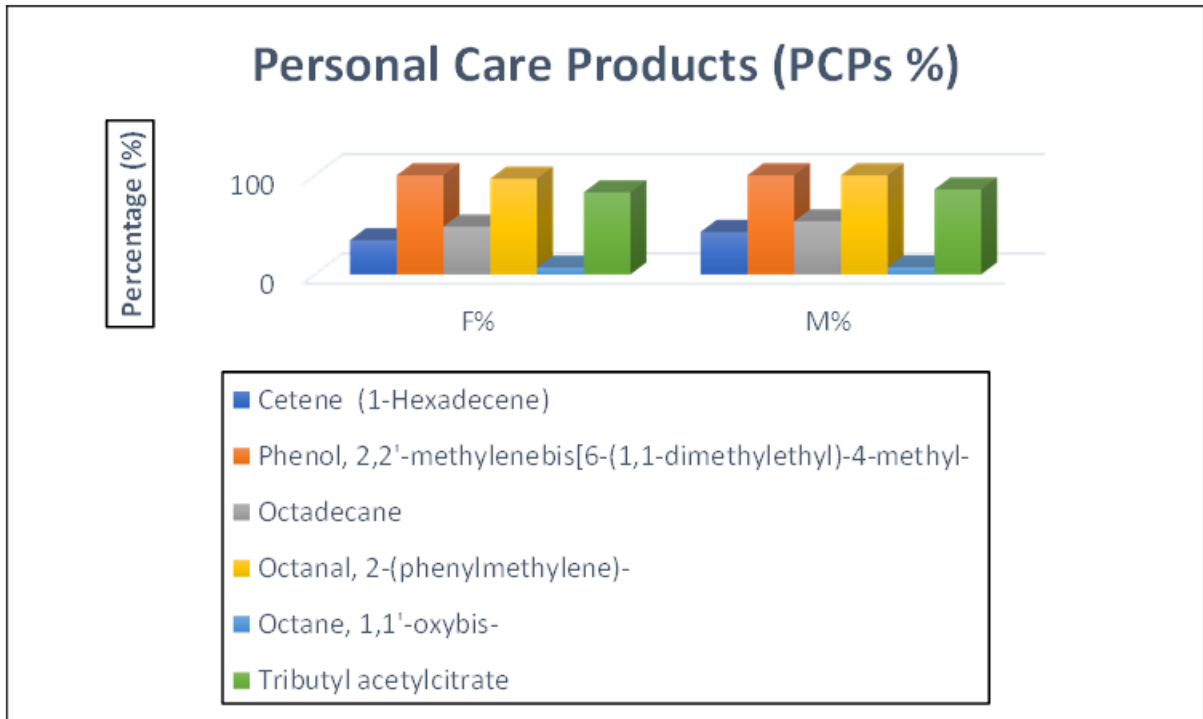
Figure 4.30: Comparison for distribution of PCPs between both sexes

Figure 4.27 shows that there is minimal disparity in PCP exposure between females and males.
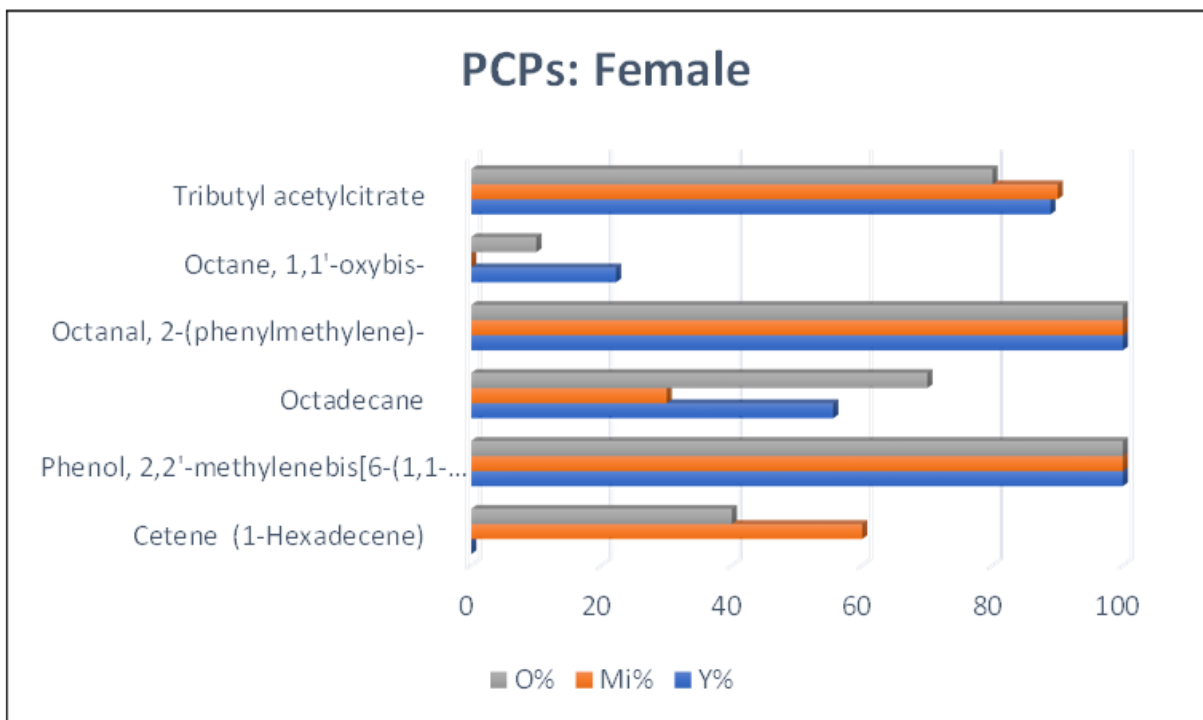
Figure 4.31: PCPs distribution among all age groups among females

The statistics in Figure 4.31 display the exposure of females to PCPs across different age groups. The data shows that there is less than 5% exposure to cetene in the young age group, but this figure increases to 60% in the middle age group. In the case of octane,1,1'-oxybis-, exposure is less than 25%, with the latter showing the lowest exposure among all the

molecules. Additionally, less than 5% exposure to some molecules is observed in the middle age group. Finally, certain molecules show 100% exposure across all age groups.
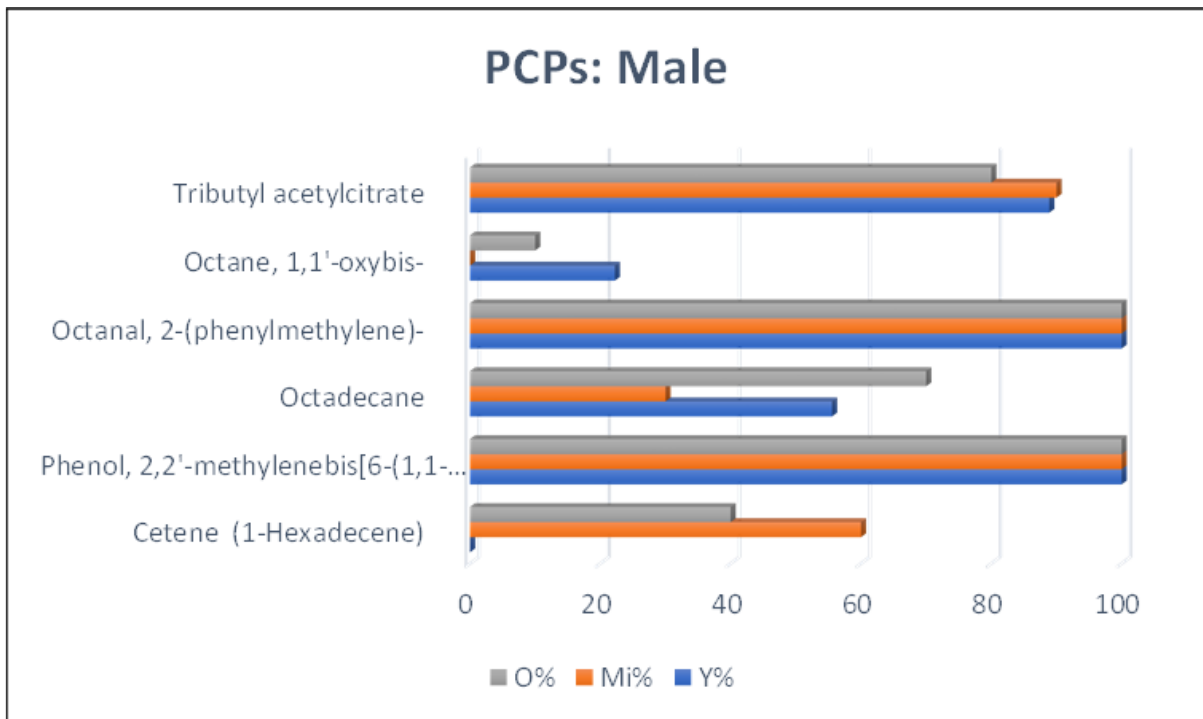
Figure 4.32: PCPs distribution among all age groups among males

Figure 4.32 shows the exposure of PCPs in males across all age groups, with over 60% exposure observed for the highest number of molecules in the elderly age group. In the middle age group, less than 5% of octane, 1,1'-oxybis-, and cetene were observed in the young age group, the exposure was also less than 5%.

5   Discussion

In the study, we performed an untargeted analysis on serum samples obtained from healthy subjects. Through this approach, we were successfully able to identify a total of 66 molecules by confirming their mass (m/z) and retention index (sec) using mass spectrometry. Since accurate identification is a critical component of the analysis, we also compared all of these molecules to the literature on exposome research to assess the quality of our data.[11, 12]

To completely understand the significance of the detected molecules in the environment, it is necessary to comprehend the nature of molecules. To accomplish this, we divided each molecule based on how it could potentially be used. Reviewing publications and looking up specific traits or uses under PubChem have been used to conduct this analysis[13, 11]. As a result, we were able to group all the molecules into nine categories, namely:- industrial chemicals, pesticides, household chemicals, and pesticides, with subclassification (e.g., p.p.-DDE is an organochlorine pesticide), personal care products, phthalates, polycyclic aromatic hydrocarbons, and other categories that are further subclassified as molecules used as food additives, volatile organic compounds (VOC), food additives, and polyfluoroalkyls (PFA). In comparison to other categories, we were able to identify a greater number of the molecules in our research as belonging to the groups that include household chemicals, industrial chemicals, pesticides with primary subclassifications such fungicides, biocide, organochlorine pesticides, and non-food pesticides with 20% each contribution and personal care products with 15% contribution among all identified exposomes. Following this, we also discovered molecules from PAH and phthalate categories, which are somewhat less numerous than those from other categories but made a significant analytical contribution because it is generally acknowledged that exposomes have a significant role in human health. Although our analysis is limited to 66 molecules we were able to isolate chemical from a variety of categories to gain a better understanding of the nature of exposomes.

Considering the first category, household chemicals, we identified many of the molecules used as flavoring or fragrance reagents in air fresheners or perfumes. Exposure to fragrances and related substances at high concentrations can lead to several health issues in individuals, including sensitivity to airborne particles, sensory irritation, and discomfort with odors. Additionally, they can negatively affect the respiratory system, which could lead to lung disorders, irregular breathing, and, in circumstances of excessive exposure, cancerous effects. Therefore, this made it easier for us to comprehend how fragrance reagents found in common household products could greatly impact the atmosphere around us.[11]

In industrial chemicals, according to our investigation, the majority of the discovered compounds are used as plasticizers in the plastic industries. and relatively few molecules come from petroleum. Plastics include compounds that can cause cytotoxicity, hormone abnormalities, and oxidative stress. Extreme exposure to these products may have negative health effects. Consequently, finding these compounds has benefits that are related to health conditions. Phthalates, another vital category in our analysis, play a crucial part when it comes to plastics and their exposure because they are thought to be the most harmful chemical additives in plastics for human health.[11]

For the exposome analysis, the primary emphasized category is pesticides. Pesticides are mainly coming from foods and the environment. Agriculture is one of the largest sources of pesticide consumption. Pesticides can be further classified as fungicides, herbicides, biocides, non-food pesticides, and organochlorine pesticides depending upon the use of chemicals. Due to their lethal nature, unintentional exposure to pesticides can pose a serious risk to humans and other living things.[12, 14] Exposure to pesticides is co-related with diseases like cancer,

hormone disruption, asthma, allergies, and hypersensitivity. The primary pesticide found in our investigation was organochlorine pesticides (p,p'-DDE), which have been included in numerous articles as a harmful toxin for human health.[15]

Many personal care items, such as deodorants, sunscreen lotions, and skin conditioning agents, are used daily. Individuals are exposed to these compounds, even in trace amounts, through various routes such as skin absorption or inhalation. Therefore, to determine the possible health danger for the whole population, it is necessary to perform a collective examination of these exposomes.

PAH contributes to 8% of molecules among all the identified exposomes. PAH is a persistent environmental molecule and is also one the most considered toxic molecules in terms of human health. The impact on human health mainly depends on the length and the route of exposure. PAH toxicity varies by different variables, mainly the age considered to co-relate with risk factors for health status. Vital molecules like anthracene, and naphthalene, impart in direct skin irritation, extreme exposure may lead to decreased immune function, and kidney and liver damage. Hence identification of these exposomes leads to understanding human health status.[15, 9, 7]

Exposomes from other category, medicine/drugs, food additives, PFAs, VOC, and organophosphorus compounds contribute 4% and 3% contribution among all identified exposomes.

According to our data, we were able to detect 30 molecules in both male and female subjects. It enabled us to proceed with the sex-based understanding differential in exposomes. When the entire population is taken into account, major exposure from household and industrial chemicals, as well as the identification of the PAH category, is observed.[9] Exposure for the category of the pesticide was predominantly observed for molecule p,p'-DDE, while the exposomes from the rest of the category were hardly detected in the overall population.[10] When looking at females and males exposure analysis, males possess principal exposure for molecules from the household category, for instance, piperine, and acetophenone. Apart from this, the female population was characterized by a bigger exposure to toxic agents. Now, to ascertain whether differences exist in the exposome between various age groups, we move on by examining comparative analysis for young, middle, and old people age groups. We observed that young age groups were primarily exposed to specific compounds found in home industrial chemicals, pesticides, and PCPs.[9] Certain compounds were found in middle-aged groups exclusively but in less than 10% of exposure. Furthermore, there was minimal variation found for molecules exposed to over 25% across all age groups. This result showed that a critical finding was the middle-aged adult group's ability to identify substances to which they had been exposed minimally. Thus yet, the findings offer little insight into exposome. It is necessary to proceed according to the category and then co-relate with sex and age to get a clear idea about the exposome behavior from each category concerning sex and all age groups.[12] To comprehend the exposome in a wide range along with plausible impact on variables like age and gender, we conducted this strategy by focusing on those categories that contribute the main portion to identified exposome molecules. Consequently, household chemicals, industrial chemicals, pesticides, and PCPs were all included.

Under the household category, except benzothiazole, which clearly shows less exposure in males as compared to females, and acetophenone, which shows less exposure in females as compared to males, we were unable to identify any variations in molecules between males and females within the household category.[14] However, altogether, not much of a difference

has been seen between them. Upon examining all age groups of both males and females, it was observed that tridecanal is efficiently identified in medium age groups for females, whereas benzothiazole did not show any notable identification in the old age group. In general, middle age groups are exposed to most molecules. However, not a single group of males was exposed to tridecanal. Benzothiazole exposure is minimal in other groups as well, but it is absent from the old age group. Significant exposure for the middle-aged group is also seen here. Therefore, considering tridecanal exposure in males and females can be a discriminating factor that further provides a clearer understanding depending on age groups.

When taking into account the industrial category, phthalic anhydride shows a clear difference in exposure between males and females, while other substances show approximately equal exposure across the sexes. When it comes to female age groups, the remaining exposomes, excluding lilial exposure, clearly demonstrate exposure in the elderly age group. Middle-aged persons are then mostly exposed to exposomes, mainly lilial exposure is predominant among them. It's interesting to note that, when compared to other age groups, lilial exposure to the younger age group exhibits a significant difference. In contrast, middle-aged males are primarily exposed to molecules, and some are from the old age group. In addition to phthalate, other molecules that should be taken into consideration when evaluating plastic exposure are lilial and phthalic anhydride. These two molecules exhibit notable differences in exposure in relation to age criteria for lilial and sex as well as age criteria for phthalic anhydride[15].

The third category, pesticides, is also unclear how exposomes are distributed based on sex. However, we observed a range of results and a scope for measuring exposure when we focused on the appropriate age criterion for females. o-hydroxyphenyl is a biocide that is promptly exposed to people of all ages and exhibits simple exposome assay detection. Only a smaller percentage of middle-aged people were found to have 1-dodecanol. The elderly population is extensively exposed to phenol, 4-(1,1-dimethylpropyl)-pesticide, while young children are primarily exposed to cotinine. The most widely used organochlorine pesticide, p,p'-DDE, is thought to be highly exposed and offers a biomarker for determining pesticide exposure. While pp-DDE has the highest exposure of all compounds across all age groups, the middle-aged male population is not exposed to o-hydroxybiphenyl and, as well as phenol, 4-(1,1-dimethylpropyl)- least noted. Exposure to 1-Dodecanol and o-hydroxybiphenyl helps differentiate the effects of pesticides according to age groups.

Personal care product exposure with respect to sex did not present well clarify data as it was expected. Age categories for females show a substantial difference in cetene exposure, with the young group being fewer exposed, the old group being less exposed, and the middle-aged group being more exposed Interestingly, increased exposure was observed in older age groups compared to younger age groups, since octadecane is mostly used for skin conditioning in cosmetics. In addition, 100% of all age groups are exposed to octanal,2-(phenylmethylene), which is also utilized as skin conditioning[14]. Octane, 1,1'-oxybis-was not found to be present in middle-aged people. Remarkably, the same information is also shown by the male exposure in all three age groups.

To sum up, it would be challenging to comprehend or gain understanding if the exposure analysis was solely based on sex. We were able to comprehend the distribution of exosomes and their effects, as well as what population is primarily affected by particular exposomes, when we further focus on male and female exposomes based on age-related parameters. Several interesting findings are revealed. Those in the middle age group who belong to the household category are more likely to be exposed. The discovery of novel ideas regarding the consideration of phthalic and lilial anydride can be a useful strategy to determine exposure to plastic.[16] The most common pesticide exposure source, p,p'-DDE, was found in both age

groups with a high percentage, indicating that behavior is not gender- or age-dependent. Surprisingly, not only do men and women exhibit the same exposure pattern to personal care products, but so do age groups. This finding refutes the notion that women are more exposed to these items than men.[16]

5.1 Strengths and Limitations

The investigation carried out on healthy persons determined the exposome in a cohort of 57 subjects exposed to the same work environment. The current study does not support any variance in exposomes when considering sex alone, but it can better describe sex between different age groups. The study only looked at two factors, age, and sex; as a result, it is difficult to understand the features of the subjects and how they relate to exposures. More characteristics will therefore be beneficial. If we also increase the population, the results might provide a compelling explanation for the sex difference.

5.2 Recommendations for Future Research

Future research can be conducted on a big scale with a variety of characteristics, such as lifestyle, habitat, alcohol intake, smoking status, habits, etc., to overcome these limitations and also improve the accuracy of the results and give a wide range of information about exposure in healthy participants.

6   References

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