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Assessing contaminant removal in wastewater using cloud-based metabolomics platform

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ABSTRACT

Pharmaceuticals and personal care products (PPCPs) are increasingly used worldly and become necessary for medicinal purposes and to enhancing quality of life. Over the three past decades are received attention as emerging pollutants due to their extensive applications and persistent emissions and capability to cause physiological effects in humans even in low concentrations. Accordingly, one of the main aims of this research was to detect for a list of PPCPs that could be existed in raw and treated wastewater in Columbia wastewater treatment plant (CWWTP) using chromatography mass spectrometry online platform (XCMS). The investigation process applied for selected putatively of 61 compounds for literatures and through the analyzing by XCMS. A total of 29 PPCPs putatively detected and listed for further analysis. The removal efficiency of PPCPs were illustrated large variability, depending on various factors such as physiochemical properties of molecules, mixing and dilution of wastewater matrices during high discharge particularly during raining seasons, retention time (RT), polarity and hydrophobicity. The results showed that the removal efficiency was variable clearly,for instant, antibiotic class recorded(-236.3 to 97.6%), for NSAID recorded (-53.9 to 84.3%),for stimulant (-6.9 to 99.7%) and other PPCPs were recorded different removal efficiencies like Ritonavir 63.6%,prednisolone 73.7%,while DEET, and Triclosan recorded, 56.3% and -81.7%,respectively.The CWWTP demonstrated prefect removal for Azithromycin, Tolfenamic acid, 1,7-Dimethylxanthine, Caffeine, Cotinine and Morphine with average > 95%.Different treatment processes including, adsorption onto solid particles, precipitation process and biological degradation govern the PPCPs removal. Different relationships and parameters were considered regarding overall removal, for instance, the direct relationship between higher (logKow) and sorption to activated sludge due to hydrophobic nature of PPCPs will increase removal efficiency of these compounds through partitioning to solid phase. Conversely, pka could have an impact on sorption and biodegradation due to the ionization state of PPCPs compounds because the signal charged the compound will show reduce in sorption of these ions to the solid particles of sludge compared to their neutral counterparts. Consequently, these interacting mechanisms and physiochemical factors, lead to a wide variability in overall removal efficiency which were already computed based on relative intensities of PPCPs ion molecules.

Keywords: pharmaceuticals and personal care products, chromatography mass spectrometry, ultra-performance liquid chromatography, untargeted analysis, MetaboAnalyst.

INTRODUCTION

The presence and widespread occurrence of synthetic micropollutants, such as pharmaceuticals and personal care products (PPCPs), even at low concentrations (ng/L–µg/L), in the environment and aquatic systems raise concerns about potential carcinogenic, mutagenic, and reproductive toxicity risks to aquatic life, animals, and humans. (Chen et al., 2023; Loganathan et al., 2023). PPCPs are commonly found as contaminants in soil, surface and groundwater, municipal sewage, and in both influent and effluent streams of wastewater treatment plants (WWTPs)

(Fu et al., 2019; Zhang et al., 2018). The presence of PPCPs in municipal wastewater has increased concerns about their negative impacts on both human health and ecosystems (Bayati et al., 2021). Despite of the environmental, health and aquatic concerns of PPCPs, limited studies have been conducted on their metabolites that could be existed inside human body and excreted out to wastewater system, the focusing was on the parent compounds and even fewer attention for the metabolites. However, studies confirmed, more than 50 % of the most consumed pharmaceuticals were excreted out of human body in form of metabolites with higher amounts comparing to the parent compound, that was result of human metabolites, as well as, this new production might be formed due to the transformation in the sewer system or limitations in the metabolite detection (Meyer et al., 2024).

The wide range of PPCPs detected in wastewater samples can be attributed to factors such as the presence of hospitals, population growth, increased use of PPCPs, local regulations, and climatic conditions (Adeleye et al., 2022). Due to human excretion or disposal expired drugs in toilets, the WWTPs considered as a main source of the PPCPs compounds in contaminating the surface and ground water (Al-Mashaqbeh et al., 2019a; Giebułtowicz et al., 2020a). There were a complexity and expansivity procedure followed to analysis of PPCPs manually, due to thousands of features that could be detected by high resolution LC/HRMS in one extract (Domingo-Almenara et al., 2018; Reyes et al., 2021; Tautenhahn et al., 2012a).Thus, the Chromatography mass spectrometry online platform (XCMS) was used for its ability to process LC–MS data and to evaluate the removal efficiency of PPCPs in the wastewater treatment plant (WWTP) based on the relative intensities of ion chromatograms.

Untargeted metabolomic analytical approach could be employed through several analytical techniques. Generally, liquid chromatography coupled with high-resolution mass spectrometry (LC/HRMS) has been frequently used for untargeted screening studies, because so many molecules might be assessed in signal analysis (Tautenhahn et al., 2012a). The untargeted analysis method works well for examining unknown metabolites in biological samples, including unidentified (Amanda Dwikarina, 2019).The main purpose of untargeted metabolomic analysis was to evaluate the deference between two group through compute the dysregulated of each feature (upregulated and downregulated) (Bayati et al., 2022). Ultra-performance liquid chromatography (UPLC) coupled with time-of-flight high-resolution mass spectrometry (TOF HRMS) was used for detection, while liquid chromatography coupled with triple quadrupole mass spectrometry (LC–MS/MS) was employed for the identification and quantification of PPCPs in raw and effluent wastewater from the Columbia wastewater treatment plant (CWWTP). UPLCs are more effective at detecting small molecules, particularly when used in conjunction with HRMS. The UPLC-HRMS is superior to traditional HPLC columns in terms of sensitivity, resolution, and peak capacity(Wang et al., 2012). While LC Triple Quad MS/MS is better suited for quantification (targeted analysis), UPLC-ToF HRMS is ideal for chemical profiling (untargeted analysis) (Amanda Dwikarina, 2019). UPLC devices were established in introduce for the latest version of modern metabolomics tools, that uses porous particles that are less than 2 μm in diameter on the inside (Amanda Dwikarina, 2019).

XCMS Online is a cloud-based informatics platform designed for processing mass spectrometry data, particularly for untargeted metabolomics. It provides a user-friendly interface and is freely accessible (Tautenhahn et al., 2012b; Gowda et al., 2014). XCMS was enhanced and developed to process paired two-group comparisons, higher-order meta-analysis, and multiple group comparisons as well as it has the ability to conducted complete data processing from raw data upload, peak detection, retention time correction calculations, profile alignment, comprehensive statistical data evaluation, and visualization to putative metabolite identification (Lu et al., 2019; Vu et al., 2020; Gowda et al., 2014).

Studies showed there are several free and accessible data analysis tool can handle MS data: MetAlign, MZmine, SpectConnect, and XCMS (Coble and Fraga, 2014a).The primary function of these tools is to analyze large volumes of instrumental data generated by LC and GC devices. The results obtained can be considered preliminary, providing an initial overview for metabolomic analysis including (filtering, peak annotation, peak detection, and peak alignment). The aim of XCMS tool is convert the large data resulted for instrumental data set into useful and usable formats that be easily analyzed and interpreted (Coble and Fraga, 2014b). Based on the literatures, there was an increasing in using XCMS online

platform in the field of environmental sciences, due to its ability in facilitating of non-targeted of environmental pollutants, pollutant transformation studies, non-targeted metabolites analysis and the analysis of metabolic impacts from pollutant exposure (Ma et al., 2022). The main outcomes that could be obtained from XCMS tool might be classified as preliminary statistics or an overview of the processed data, and further statical process will be used to get the final results that would provide a meaningful indicator for take the suitable decision and provide appropriate recommendations. The 61 PPCPs compounds that were selected for this research collected from previous studies (Al-Mashaqbeh et al., 2019b; Bayati et al., 2021). The raw data that was extracted from UPLC analysis were uploaded to XCMS platform for analyzing, pairwise-job was run after a process of filtration to detect the PPCPs compounds and scanning in METLIN library. The specific aims of this study were to: 1) screening for a list of compounds that could be present in raw and treated wastewater by using XCMS and the METLIN library then to determine the removal efficiency of each compound, 2) statistical view of the data uploaded to XCMS to get an overview about the prevalence and fate of PPCPs in raw and treated wastewater, 3) using an advance statistical tool MetaboAnalyst to assess the variation between the intensities of the PPCPs compounds in raw and treated wastewater. The results could provide a better understanding about the type and number of PPCPs that was existed in wastewater and estimate the efficiency of conventional wastewater treatment system in removing these chemical compounds, as well to enhance the removal capacity by suggesting an advanced treatment mechanisms for the persistence compounds.

MATERIALS AND METHODS

Chemicals

Methanol, formic acid and acetonitrile (HPLC-grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Sample collection

Samples were collected in October 2018 from both raw wastewater (influent) and treated wastewater (effluent). Influent samples were taken before the primary settling tanks prior to the treatment process, while effluent samples were collected on the same day after the secondary settling tanks. Samples were collected in 1000 mL polypropylene bottles. During transportation to the University of Missouri laboratory in Columbia, samples were maintained at low temperatures (0–3 °C). Upon arrival, all samples were stored at −20 °C until analysis.

Analytical methodology

Wastewater samples were collected in triplicate using 50 mL polypropylene centrifuge tubes. The samples were first vortex for 15 seconds before being transferred to smaller tubes to be homogenized using Vortex Genie 2, Fisher, NY, USA. A 1.8 mL aliquot was then placed into 2 mL microcentrifuge tubes and centrifuged (Eppendorf 5415D, Hamburg, Germany) at 12,000 rpm for 15 minutes. Following centrifugation, 1.5 mL of the clear supernatant was transferred to 5 mL glass tubes and mixed with an equal volume of methanol. After brief vortex mixing (10 seconds), 1.5 mL of the extract was filtered using a 0.2 µm PTFE syringe filter (Acrodisc, Waters, MA, USA). The filtered extracts were stored at −20 °C prior to analysis by LC–MS/MS.

In this study, the non-targeted screening (NTS) approach that was used for detection of the PPCPs compounds in raw and treated wastewater, to estimate the removal efficiency of CW-WTP regarding PPCPs that was investigated. LC–MS analysis was carried out using a Bruker maXis impact quadrupole time-of-flight mass spectrometer interfaced with a Waters ACQUITY UPLC system. Chromatographic separation was performed on a Waters BEH C18 column (2.1 × 100 mm, 1.7 µm particle size). The mobile phase consisted of eluent A (0.1% formic acid) and eluent B (acetonitrile), with a linear gradient from 95:5 to 30:70 (A:B) over 30 minutes. From 30 to 33 minutes, the gradient was increased from 70% to 95% B and held at 95% B for 3 minutes. The composition was then returned to 5% B and maintained from 36 to 40 minutes. The flow rate was set at 0.56 mL/min, and the column temperature was maintained at 60 °C. Mass spectrometric detection was conducted in either negative or positive electrospray ionization mode. The operating parameters included a nebulizer gas pressure of 43.5 psi, a dry gas flow of 12 L/min, a drying temperature of 250

°C, and a capillary voltage of 4000 V. Data were acquired over an m/z range of 100–1500 and subsequently auto-calibrated using sodium formate after acquisition. UPLC–MS ion chromatograms were uploaded to the XCMS platform for deconvolution and data processing, supported by the Center for Metabolomics at the Scripps Research Institute. Data processing steps, including peak detection, grouping, spectral extraction, and retention time alignment, were carried out using XCMS algorithms. The resulting spectra were then annotated, and compounds were identified and classified through integration with the METLIN database, one of the largest repositories of metabolite information. The METLIN Database that was connected to the XCMS online platform makes it simple for us to identify unknown metabolites. Based on the precise mass, METLIN generates a list of possible metabolite identities (Dwikarina, 2019)

In this study, an untargeted metabolomic approach was used to generate information on the analyzed compounds. Data obtained from the XCMS online platform included total ion chromatograms (TIC), metabolomic cloud plots, and non-metric multidimensional scaling (NMDS) analyses. The PPCP compounds selected for this research were collected from the literature and are listed in Table 1 for subsequent analysis using XCMS and MetaboAnalyst.

Table 1. List of PPCPs compounds elected from literature

No.	Compound	Category	No.	Compound	Category
1	Triclocarban	Antibiotic	32	Paracetamol	NSAID
2	Lincomycin	Antibiotic	33	Valsartan	Angiotensin II receptor antagonist
3	Trimethoprim	Antibiotic	34	Furosemide	Diuretic
4	Azithromycin	Antibiotic	35	Pravastatin	Statins
5	Erythromycin	Antibiotic	36	Atorvastatin	Statins
6	Sulfamethoxazole	Antibiotic	37	Atenolol	β-blocker
7	Clarithromycin	Antibiotic	38	Metoprolol	β-blocker
8	Flucloxacillin	Antibiotic	39	Propranolol	β-blocker
9	Thiabendazole	Antibiotic	40	Warfarin	Anticoagulant
10	Clotrimazole	Antibiotic	41	Dipyridamole	Platelet Inhibitors
11	Miconazole	Antibiotic	42	Diphenhydramine	Antihistamine
12	Ritonavir	Antiretroviral	43	Meclizine	Antihistamine
13	Fluoxetine	Antidepressant	44	Metoclopramide	Antiemetic
14	Norfluoxetine	Antidepressant	45	Cimetidine	Anti-Acid Reflux
15	Oxazepam	Antidepressant	46	Triclosan	Antiseptic
16	Sertraline	Antidepressant	47	Prednisolone	Synthetic Glucocorticoid
17	Primidone	Antiseizure	48	Tribenoside	Vasoprotective
18	Carbamazepine	Antiseizure	49	Clopidogrel	Antithrombotic Agents
19	Phenytoin	Antiseizure	50	Iopamidol	Contrast Agent
20	Propyphenazone	Analgesic	51	Estrone	Estrogenic hormone
21	Salicylic acid	NSAID	52	DEET	Insecticide
22	Carprofen	NSAID	53	Tetracaine	Local anesthetic
23	Fenoprofen	NSAID	54	1,7-Dimethylxanthine	Stimulant
24	Flurbiprofen	NSAID	55	Amphetamine	Stimulant
25	Ibuprofen	NSAID	56	Acetaminophen	Analgesic
26	Indomethacin	NSAID	57	Caffeine	Stimulant
27	Indoprofen	NSAID	58	Cotinine	Stimulant
28	Ketoprofen	NSAID	59	Methylenedioxymethamphetamine	Abuse drag
29	Naproxen	NSAID	60	Morphine	Narcotic analgesic
30	Tolfenamic acid	NSAID	61	Sulfamethazine	Antibacterial
31	Phenazone	NSAID			

XCMS workflow and analyzing step

The LC–HRMS data were uploaded to XCMS for analysis. The workflow and analysis steps are described in Figure 1.

Data processing using XCMS online

Biological and environmental samples are challenging to handle, particularly due to the wide range of compounds they contain. Analysis of these samples using chromatography coupled with high-resolution mass spectrometry generates large volumes of data, including mass-to-charge ratio (m/z), retention time (RT), and peak intensity information, which require significant time and effort to process. Therefore, the use of specialized software for mass spectrometry data processing is essential for accurate identification and analysis. Chromatography mass spectrometry, is one of the widely used data-processing tools. It is an efficient, accurate, and freely accessible platform that has been extensively applied in environmental science for processing MS data (Yang et al., 2025). The data obtained from the UPLC–HRMS system were uploaded to the XCMS Online platform and processed using the pairwise job workflow. The XCMS algorithm converted the data files and enabled peak detection, peak grouping, and the determination of relative intensities and retention times (RT). The feature's table that was resulted from running a pairwise-job was the preliminary target. Detection process for PPCPs started by filtration by name for all the 61 compounds that was selected for literatures, then we selected an advance filtering process based on

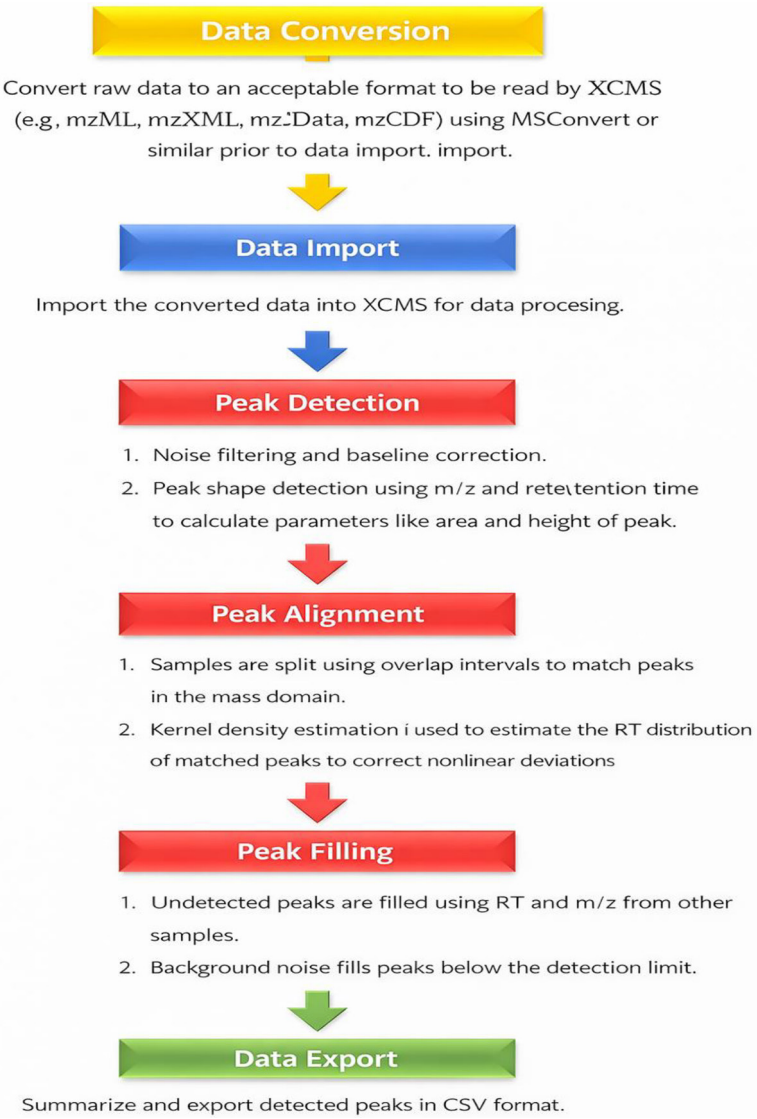

Figure 1. General workflow of XCMS

some criteria were set up included (fold change ≥ 1.5, p-value ≤ 0.05, maxint ≥ 2000, 800 ≥ mzmed ≥ 125). Identification of PPCPs compounds in raw and effluent samples was performed using the global metabolomic XCMS Online with integration of METLIN database. Table 2 lists 29 candidate PPCP compounds identified in this study. XCMS plays a vital role in providing statistical analysis and generating several visual outputs, including principal component analysis (PCA), total ion chromatograms (TIC), cloud plots, extracted ion chromatograms (EIC), and mass spectra (m/z). Each detected feature was systematically examined in XCMS Online and putatively annotated using the METLIN database, which contains over 960,000 metabolites.

Statistical analysis

MetaboAnalyst version 6.0 was the last updated of this platform, which was started in 2009 with (v 1.0) and ended in 2024 with (v 6.0). Unified workflow of MetaboAnalyst was for raw spectra processing, statistical analysis and functional interpretation. Thus, was used for statistical analysis to illustrate the variation between the intensities of the PPCPs compounds in raw and treated wastewater, from all the modules available in this platform including (Spectra Processing [LC-MS w/wo MS2], Functional Analysis [LC-MS] …etc.). Statistical Analysis [one factor] module was used in analyzing in this study. PCA and heatmap visualization outcomes that was resulted from the analysis, which was represent the behaviors of PPCPs compounds in raw and treated wastewater. MetaboAnalyst was performed to assess the variation between the intensities of the PPCPs compounds in raw and treated wastewater. Pairwise comparison was processed by using MetaboAnalyst (v 6.0) with significant differences test 95% confidence level (p-Value ≤ 0.05). The average removal efficiencies and standard deviations of data were calculated by using Microsoft Excel 2021.

Removal efficiency

The removal efficiency (%R) for each compound detected in raw and treated wastewater was determined based on the relative intensities using XCMS Online. The calculation was based on the relative intensities of each compound in the raw and treated wastewater samples analyzed in triplicate. The following equation was applied for the calculations.

$$R\,(\%) = \frac{Intensity\,(influnt) - Intensity\,(effluent)}{Intensity\,(influent)} \times 100 \quad (1)$$

where: R (%) – removal efficiency percentage provides a high-throughput measure of how effectively the treatment process reduces the compound's relative abundance, *Intensity* (*influent*) – the exposure level of each compound in raw wastewater as a relative intensity, *Intensity* (*effluent*) – the exposure level of each compound in treated wastewater as a relative intensity.

Then the average removal efficiency was calculated for the triplicated removal efficiencies.

RESULTS AND DISCUSSION

Untargeted analyses

A non-targeted screening approach was employed to detect PPCPs using XCMS. Of the 61 compounds initially investigated, 29 compounds exhibited high signal intensities in both influent and effluent wastewater samples (Table 2) following UPLC-HRMS analysis and data processing through the XCMS online platform. A pairwise job was conducted in XCMS Online, where the data files were processed using XCMS algorithms for peak detection, peak grouping, spectral extraction, and retention time (RT) alignment and correction. A two-group comparison approach was applied in this study, which is one of the most commonly used methods, based on comparing two conditions such as "before" and "after" or "control" and "treatment." In this case, the statistical comparison was conducted between raw (influent) and treated (effluent) wastewater from the CWWTP, based on the relative ion intensities in both positive and negative ionization modes.

Several visual outputs were generated by XCMS, including total ion chromatograms (TIC) and cloud plot analyses. Figure 2 present the original and corrected TICs for both positive and negative ionization modes. TIC correction was applied to all detected ions in both modes based on their relative intensities and retention times. The results indicated no significant retention time deviation for most peaks, demonstrating that the UPLC-HRMS analysis provided stable

Table 2. List of 29 PPCPs identified from XCMS analysis

No.	Compound	Category	No.	Compound	Category
1	Trimethoprim	Antibiotics	16	Tolfenamic acid	NSAID
2	Azithromycin	Antibiotics	17	Atenolol	β-blocker
3	Sulfamethoxazole	Antibiotics	18	Dipyridamole	Platelet inhibitors
4	Clarithromycin	Antibiotics	19	prednisolone	Synthetic glucocorticoid
5	Ritonavir	Antiretroviral	20	DEET	Insecticide
6	Fluoxetine	Antidepressant	21	1,7-Dimethylxanthine	Stimulant
7	Oxazepam	Antidepressant	22	l-Amphetamine	Stimulant
8	Primidone	Antiseizure	23	p-Acetamidophenol	Analgesic
9	Carbamazepine	Antiseizure	24	Caffeine	Stimulant
10	Phenytoin	Antiseizure	25	Cotinine	Stimulant
11	Salicylic acid	NSAID	26	3,4-Methylenedioxymethamphetamine (MDMA)	Abuse Drag
12	CARPROFEN	NSAID	27	Morphine	Narcoticanalgesic
13	(±)-Flurbiprofen	NSAID	28	Miconazole	Antibiotic
14	(±)-Ibuprofen	NSAID	29	Triclosan	Antiseptic
15	(S)-Naproxen	NSAID	30		

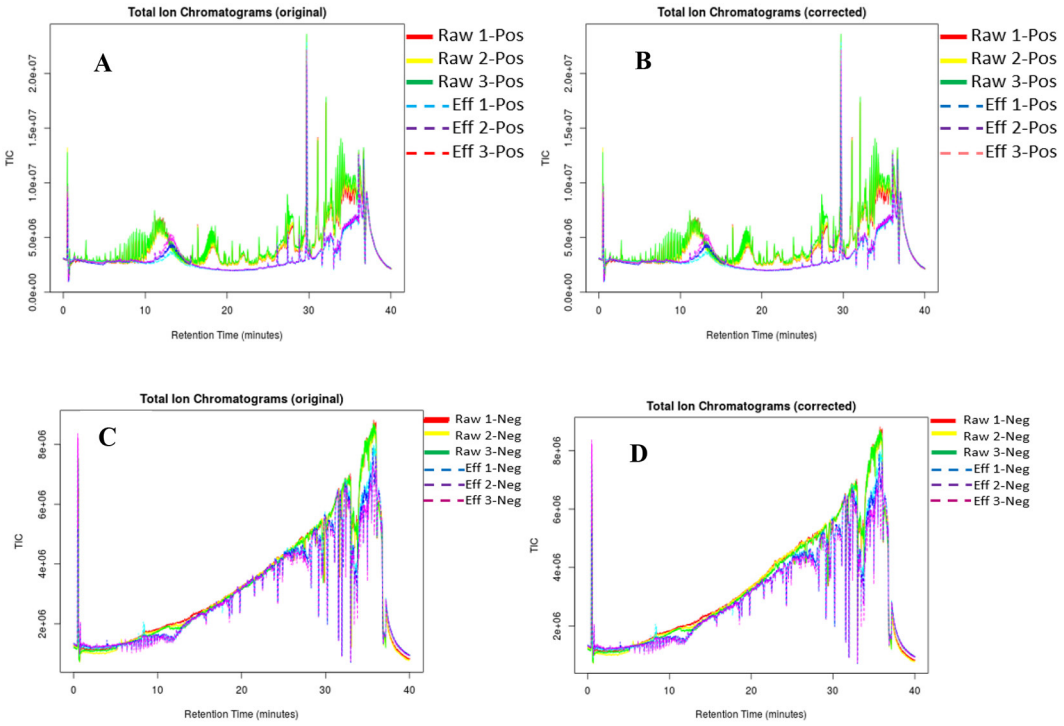

Figure 2. Original and corrected total ion chromatograms (TIC) of PPCPs in positive ion mode (A, B) and negative ion mode (C, D) analyzed using XCMS online

metabolite profiling and that the generated metabolomic data were reliable.

Figure 3A and 3B also present metabolomic cloud plots of PPCPs in positive and negative ionization modes, respectively. The cloud plot generated by XCMS (default output) displays dysregulated features in the m/z versus retention time (RT), based on predefined statistical thresholds (p-value < 0.001 and fold change > 1.5). Ion intensities were compared between the two groups (influent and effluent). In the plot, green circles located in the upper region represent upregulated features, while red circles in the lower region indicate downregulated features. The size of each bubble reflects the log fold change between raw and effluent samples, and the color intensity

corresponds to the significance level, with darker shades indicating smaller p-values. The cloud plot results for the positive ion mode showed that compound intensities were higher in the raw samples compared with the effluent, indicating degradation of compounds during the treatment process. In contrast, the negative ion mode revealed that most features were more prominent in the effluent than in the influent, suggesting incomplete removal of certain compounds or the formation of transformation products during treatment.

Figure 4 shows the non-metric multidimensional scaling result of metabolites of PPCPs in positive ion mode. The results in red color refer to raw wastewater, which were illustrated noticeable reflection, this indicates the influent wastewater massively heterogenous due to the presence of a large diverse in number of PPCPs and their metabolites because of the different sources that consisted the wastewater as well as due to mixing and dilution of wastewater matrices during high discharge particularly during raining seasons. In contrast, the clearly clustering of (effluent-pos) presented in blue color indicted relative homogenization of the chemical profile and proved that there was treatment occurred and showed partially removal happened, in same time existed of clustered signals in effluent considered a clear indication of limitation in removing of PPCPs by wastewater treatment plant.

Figure 5 represents the non-metric multidimensional scaling result of metabolites of PPCPs in Negative ion mode. Illustrated that the effluent intensities of wastewater were tightly clustered and that's refer to good removal achieved compared with influent in same ion mode, where the clearly clustering of intensities of raw wastewater refers to risky due to the highly number of PPCPs compounds that could be cared out in wastewater.

Briefly, the NMDS used as a tool for dimensionality reduction, which work to convert the thousands of peeks resulted from XCMS to a clearly dot plot, the separation and clustering of results on the both sides of scheme, clearly indicates removing occurred for specific PPCPs.

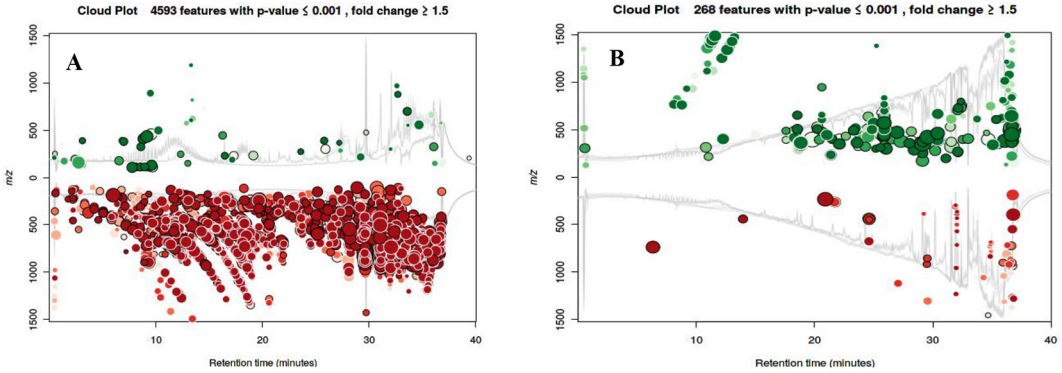

Figure 3. Metabolomic cloud plot of PPCPs in positive ion mode (A) and negative ion mode (B) analyzed using XCMS online

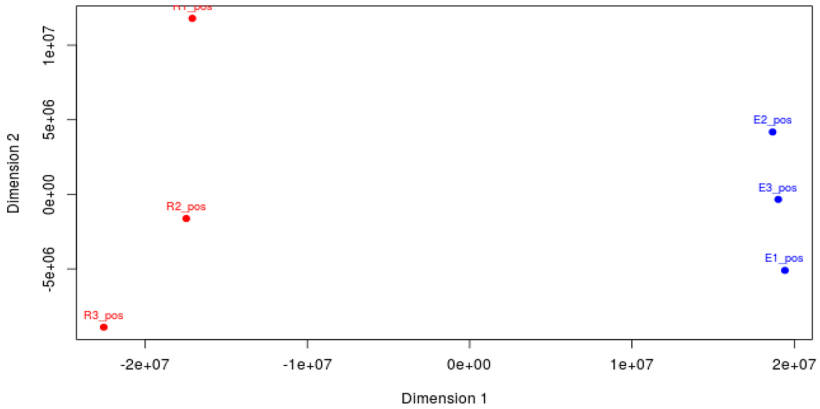

Figure 4. Non-metric multidimensional scaling result of metabolites of PPCPs in positive ion mode, clustered by each group: raw-pos (red), effluent-pos (blue) analyzed using XCMS online

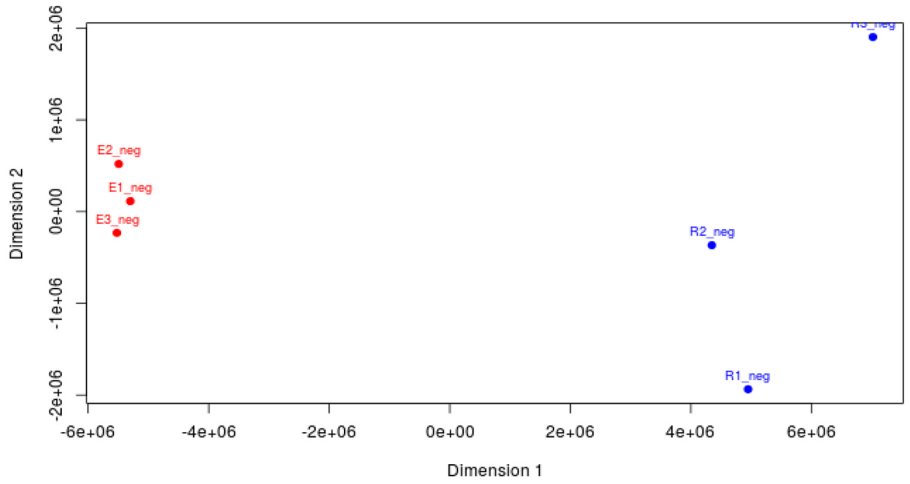

Figure 5. Non-metric multidimensional scaling result of metabolites of PPCPs in negative ion mode, clustered by each group: effluent-neg (red), raw-neg (blue) analyzed using XCMS online

Statistical analysis

MetaboAnalyst 6.0 was used to conduct PCA, applying one factor module. Figure 6 shows the PCA results in raw and treated wastewater based on relative intensities. Normalization procedure was applied, which was consist of three categories sample normalization, data transformation and data scaling. The results showed the relative intensities' abundance of PPCPs in raw and treated wastewater had a significant variance, whereas those recorded (97.2%) were in the first principal component (PC 1) and (2.4%) in the second principal component (PC 2); the compound profiles in PCA illustrated a clear separation between raw and treated wastewater, which indicates the significant difference in relative intensities of PPCPs and their metabolites before and after treatment, the triplicate sample analyzed demonstrated closely clustering of influent and effluent group, that's could refer to high similarity of PPCPs metabolites profiles and the colored circles represented that. These findings that resulted from PCA could be further supported by heatmap analysis. Figure 7 presents a heatmap illustrating the clustering of 27 chemical profiles, highlighting the most significant compounds identified using a t-test ($p < 0.001$). Nineteen compounds exhibited high relative intensities in raw wastewater in most of the triplicated samples analyzed compared to the relative intensities in treated wastewater; that indicates the achieving of clear removal efficiency by the (CWWTP) for these compounds. The last five compounds illustrated in Figure 7 demonstrated low relative intensities in raw wastewater for all the triplicate samples analyzed, while the relative intensities clearly increased; that indicates a reverse treatment and resulted in negative removal efficiency.

Removal efficiency

The removal efficiency was evaluated by comparing the relative intensities of PPCPs detected in raw and treated wastewater from the

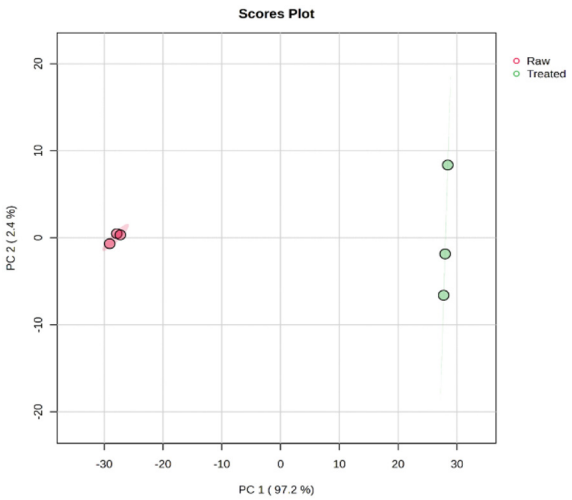

Figure 6. Principal component analysis (PCA) results of identified PPCPs compounds. Red represents the relative intensities in raw wastewater and the corresponding compounds represented in green refers to treated wastewater. Red represents lower relative abundance, while blue represents higher relative abundance in the PCA score plot. Circles of the same color represent replicates of metabolic profiles within each group, while the colored ellipses indicate the 95% confidence regions for each group

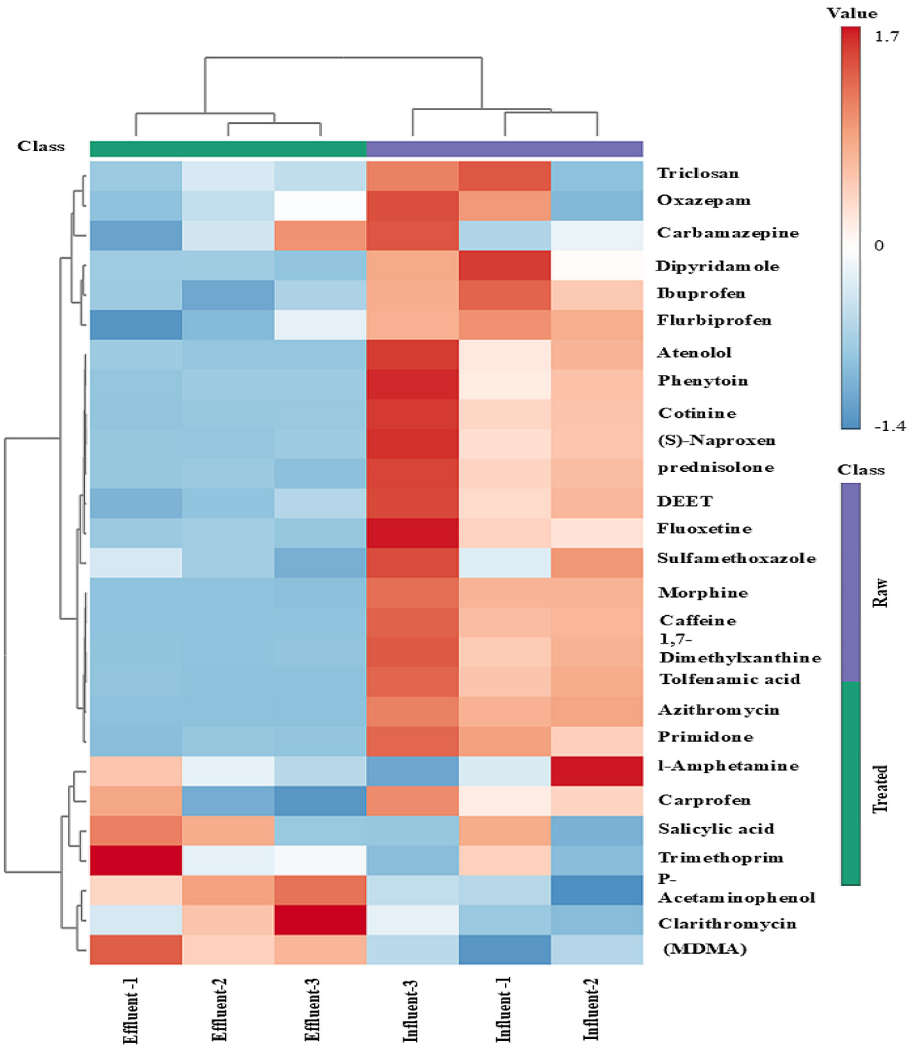

Figure 7. Heatmap of the relative intensities of the identified PPCPs compounds found in raw and treated wastewater of CCWWP. Blue represents low relative intensity, whereas red represents high relative intensity. Purple represents the compounds detected in raw, whereas green represents the compounds detected in treated wastewater

(CWWTP) (Table 3, Figure 8). The data was processed by using XCMS platform which has the ability to extract the chromatographic features from LC/MS data obtained from UPLC-HRMS device. The untargeted screening approach deemed in this study as analysis method, thus, the removal calculation will be relied on the relative intensities, rather than concentrations, the calculation was relied on the change in relative signal intensity between influent and effluent. The results indicated that the removal efficiency of PPCPs ranged from near-complete removal to minimal removal, and in some cases exhibited negative values, depending on the specific compound and the treatment processes applied at the wastewater treatment plant (WWTP) (Fernández-López et al., 2016; Giebułtowicz et al., 2020b; Verlicchi et al., 2012). This variability in removal efficacies obtained among the 29 PPCPs compounds investigated, might be justified due to different parameters including chromatographic parameter like retention time (RT), which is provide indirect information about PPCPs compounds polarity and hydrophobicity in LC/MS analysis. Compounds with longer (RT) values in reversed-phase chromatography usually have higher hydrophobicity and may have higher logKow values. As a result, these PPCPs might have a stronger affinity for sludge and a higher rate of removal by sorption procedures. On the other hand, compounds that elute at lower RT values are typically more polar and thus more persistent in the aqueous phase(Liu et al., 2022; Wang et al., 2024a; Wydro et al., 2024; Zheng et al., 2025).

Table 3. Removal efficiency for the PPPCs based on the relative intensities

No.	Compound	Avg intensity (raw)	Avg intensity (treated)	Avg R (%)	SD
1	Trimethoprim	14283	28568	-128.6	52.5
2	Azithromycin	39391	950	97.6	0.1
3	Sulfamethoxazole	4856	2769	37.5	30.9
4	Clarithromycin	5053	7705	-51.7	27.6
5	Ritonavir	1778	659	63.6	4.9
6	Fluoxetine	4440	1351	67.1	11.1
7	Oxazepam	3076	1753	15.1	75.2
8	Primidone	5740	2247	60.2	6.9
9	Carbamazepine	68361	61978	9.9	5.8
10	Phenytoin	10690	2147	78.6	5.5
11	Salicylic acid	4123	5697	-53.9	82.6
12	Carprofen	1900	1199	32.8	51.2
13	Flurbiprofen	7738	4316	43.9	16.7
14	Ibuprofen	3628	2453	32.3	4.6
15	(S)-Naproxen	6872	1740	73.4	6.0
16	Tolfenamic acid	9710	1488	84.3	3.5
17	Atenolol	4982	1043	77.2	9.3
18	Dipyridamole	2241	417	78.9	10.8
19	Prednisolone	5049	1259	73.7	8.0
20	DEET (diethyltoluamide)	9872	4298	56.3	1.3
21	1,7-Dimethylxanthine	225133	2678	98.8	0.6
22	l-Amphetamine	78360	74671	-6.9	40.0
23	p-Acetamidophenol (Acetaminophen, Tylenol)	760	1932	-216.8	207.0
24	Caffeine	4205285	10579	99.7	0.0
25	Cotinine	80714	2373	97.1	1.4
26	3,4-Methylenedioxymethamphetamine (MDMA)	190742	278055	-48.3	32.4
27	Morphine	38783	1179	96.8	1.4
28	Miconazole	404	1308	-236.3	87.1
29	Triclosan	3960	1245	-81.7	285.1

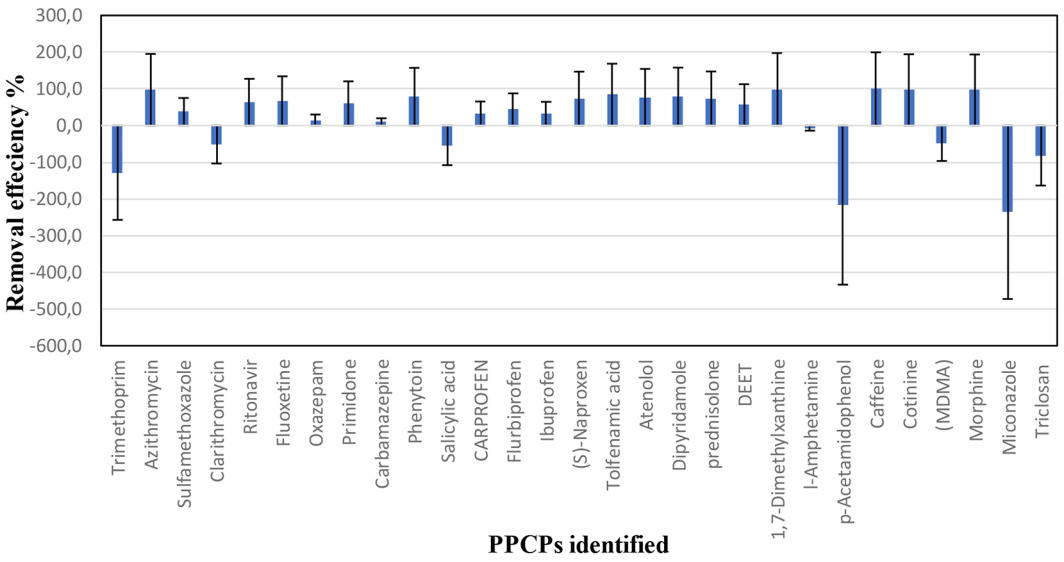

Figure 8. Average PPCP removal efficiencies based on the relative intensities

Physicochemical properties as well considered as influence parameters on removal efficiency of PPCPs compounds. Parameters such as molecular wight (MW), the octanol-water partition coefficient (LogKow) and the acid dissociation constant (Pka) represent a crucial role in evaluating the situation and fate of PPCPs in wastewater. The direct relationship between higher (logKow) and sorption to activated sludge due to hydrophobic nature of PPCPs will increase removal efficiency of these compounds through partitioning to solid phase. In contrast, the lower removal efficiency result from inverse relationship between the higher polarity and low (logKow) of PPCPs that's causing remain these compounds suspended in aqueous phase. From the other hand, (pka) could have an impact on sorption and biodegradation due to the ionization state of PPCPs compounds, because the signal charged the compound will show reduce in sorption of these ions to the solid particles of sludge. This relationship mentioned had been reported as key factor affected on the removal efficiency of WWTPs (Min et al., 2024; Wang et al., 2024b; Wydro et al., 2024). The results showed that the removal efficiency was variable clearly, antibiotic class recorded (-236.3 to 97.6%),for antidepressant and antiseizure drugs (9.9–78.6%), for NSAID recorded (-53.9 to 84.3%),for stimulant (-6.9 to 99.7%) and other PPCPs were recorded different removal efficiencies like Ritonavir 63.6%, Atenolol 77.2%, Dipyridamole 78.9%,prednisolone 73.7%,while DEET, p-Acetamidophenol, 3,4-Methylenedioxymethamphetamine and Triclosan recorded, 56.3%,-216.8%,-48.3% and -81.7%,respectively. The CWWTP demonstrated prefect removal efficiency for Azithromycin, Tolfenamic acid, 1,7-Dimethylxanthine, Caffeine, Cotinine and Morphine with average > 95%. Negative removal efficiencies have been reported in the literature due to several factors, including the reconversion of metabolites to parent PPCPs during primary and secondary treatment through the cleavage of glucuronide conjugates. Additionally, resuspension and release of PPCPs previously accumulated in sludge or sorbed onto solid particles can occur, particularly during high-flow conditions such as storm events, when increased discharge rates enhance mobilization (Giebułtowicz et al., 2020c; Kumar et al., 2022; Luo et al., 2014; Watkinson et al., 2007).

CONCLUSIONS

The XCMS online platform is a user-friendly and efficient tool with an intuitive graphical interface. Its integration with the METLIN database enhances the analysis of untargeted metabolomic LC–MS data. METLIN could generate a list of possible metabolite identities according to the exact mass. Raw LS/MS data were uploaded to XCMS online platform, pairwise job was run to estimate the removal efficacy of Columbia wastewater treatment plant. A total of 61 PPCP compounds were initially investigated, of which 29 were identified as final candidates. The selection involved filtering by compound name, followed by advanced filtering based on molecular weight and predefined criteria, including fold change $\geq$ 1.5, p-value $\leq$ 0.05, maximum intensity $\geq$ 2000, directional change (up/down), and an m/z range of 125 $\leq$ mzmed $\leq$ 800. A two-group comparison approach was applied in this study. The statistical results obtained from XCMS and MetaboAnalyst showed a significance difference between raw and treated wastewater. The comparison between raw and effluent wastewater from CWWTP was based on the relative intensities of ions in both positive and negative modes. The results showed considerable variability in removal efficiency, indicating that the (CWWTP) was not effective in completely removing the 29 PPCPs detected in the influent and effluent samples. Eleven compounds exhibited high removal efficiencies (73.4–99.7%), while ten compounds showed moderate removal (9.9–63.6%). In contrast, eight compounds displayed negative removal efficiencies.

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