



## Advanced Chromatographic Approaches for the Comprehensive Analysis of Emerging Contaminants in Wastewater Effluents

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### Abstract

This study explores the wastewater analysis of contaminants of emerging concern (CECs) in wastewater effluents using Liquid Chromatography (LC), Supercritical Fluid Chromatography (SFC), and Comprehensive Two-Dimensional Gas Chromatography (GC×GC) with derivatization, all coupled to high-resolution mass spectrometry (HRMS). These methods facilitated the identification of over 300 compounds, including frequently overlooked highly polar and non-pharmaceutical CECs. The findings demonstrate that correlation clusters in effluent samples can differentiate between easily degradable and persistent compounds, associate rain-influenced CECs with transformation products from household sources, and identify industrial contributions as source and event origins.

### Introduction

The landscape of chemical usage in Europe is vast and continuously expanding, with over 100,000 chemicals in current circulation and more compounds introduced to the market every day. This comes with significant environmental consequences, as the majority of these substances eventually find their way into the aquatic environment, where they undergo transformations, ending up as numerous unknown compounds [1,2]. Nowhere is this more evident than in wastewater, where thousands of chemical compounds are discharged, posing a challenge for environmental management. Despite the extensive monitoring efforts, less than 10% of the observed wastewater toxicity can be directly linked to CECs identified through chemical analysis [3]. This underscores the need for a paradigm shift in water monitoring strategies. Instead of solely focusing on a limited set of legacy chemicals, there is a growing attention to adopt a more holistic approach, shifting the focus towards understanding complex chemical mixtures by non-target screening [4]. Central to this transition is the careful selection of analytical platforms. While conventional methods like reversed phase LC are widely used due to their versatility in covering a broad range of water-relevant compounds, they fall short in detecting very polar and volatile substances, which can be responsible for toxicity [5]. Hence, chromatographic techniques focusing on polar compounds such as SFC, IC, and HILIC are gaining attention for their potential to address these limitations. Strategies for analyzing volatile and semi-volatile compounds by non-target screening in water remain rare [6]. The objective of this study was to establish correlations within and between chemical and meta data (i.e. operational and monitoring data) of two wastewater treatment plants (WWTPs),

with the goal of pinpointing potential sources and events occurring within wastewater effluent that drive CEC discharge. The study employed a combination of analytical techniques (LC, SFC and GC×GC) to generate comprehensive fingerprints.

### 1. Material and Methods

Effluent samples were taken flow proportional as 4-hour composite samples at different times and days between April and June 2021 for two WWTPs in Denmark (51 effluent samples in total). In addition, two 24-hour composite influent samples were taken from each WWTP. The catchment area of WWTP 1 was a more rural area, with the influence of landfill and industrial sites. WWTP 2 was in the catchment area of the capital city Copenhagen. The samples were filtered and enriched 50 times with a multi-layer solid phase extraction, described previously [2]. The MeOH extract was directly injected for analysis using both LC and SFC. For GC×GC, each sample was derivatized with MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide) following previously optimized reaction conditions [7]. All three platforms were equipped with quadrupole time-of-flight (QTOF) mass spectrometers. A summary of the analytical and data processing methods for the three platforms is provided in Table 1, with detailed descriptions available in prior publications [8,9].

A combination of non-target and suspect screening of wastewater relevant compounds was conducted via LC, SFC and GC×GC with derivatization. All three platforms were connected to quadrupole time of flight (QTOF) mass spectrometers. In Table 1 the analytical and data processing methods are summarized for the three platforms.

Table 1: Methods and software for identification of suspect compounds on the respective chromatographic platforms.

	LC	SFC	GCxGC with derivatization
Chromatographic columns	Intensity Solo 1.8 C-2, 100 x 2.1 mm column (Bruker, Hamburg, Germany)	Acquity UPC2 BEH column (3 mm x 100 mm and 1.7 µm pore size) (Waters, Milford, MA)	Zoex ZX2 cryogen-free modulator (Zoex Corporation, Houston, TX, USA); first dimension: Rxi-5Sil MS column (60 m, 0.25 mm i.d., 0.25 µm film thickness), second dimension: Rxi-17Sil MS column (1.5 m, 0.18 mm i.d., 0.18 µm film thickness) (Restek, Bellefonte, Pennsylvania, U.S.A)
Instrument	Elute HT LC QTOF: Impact 2 QTOF equipped with VIP-HESI ion source (Bruker, Hamburg, Germany)	Acquity UPC2 G2-Si Synapt QTOF with ESI (Waters, Milford, MA)	Agilent 7890B GC coupled to an Agilent 7200 Accurate Mass QTOF with EI (Agilent Technologies, Palo Alto, CA, USA)
Software data processing	TASQ 2021b (Bruker, Hamburg, Germany)	MSDial (version 4.90 <a href="https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4449330/">https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4449330/</a> )	MassHunter (Agilent version: B.07.02.1938 and GC-Image LLC (version: 2.9R1.1)
Suspect library	Targetscreener TOX and Pesticide Database v4.0 (Bruker, Hamburg, Germany) database and in-house database, where a reference standard has previously been analyzed on the same setup	STOFF-IDENT database with MS/MS data from MoNA or generated in-silico (CFM-ID 4.4.7)	NIST library

## 2. Results and Discussions

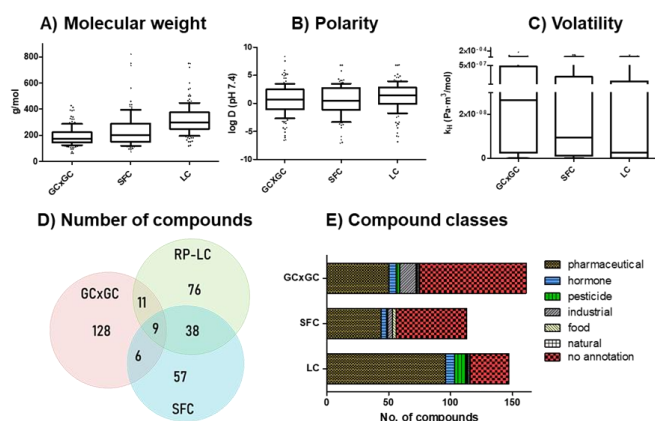
### 2.1. Chemical characterization of samples

A total of 336 unique CECs were detected in the effluent wastewater samples by at least one of the three analytical methods. 154 targets and suspects were detected by GCxGC-HRMS, 134 by RP-LC-HRMS and 110 with SFC-HRMS. The methods with most detections in common were LC and SFC where 38 CECs were detected with both methods. In contrast, GCxGC provided the most unique information as only 15 and 20 CECs were in common between GCxGC and RP-LC and SFC, respectively (Figure 1D). The confidence level was 1 for 139 compounds (i.e. analytical standard available) and confidence level 2 for the remaining compounds. For all three platforms, the suspect libraries/databases included water relevant compounds. However, different libraries, operational conditions, thresholds and data processing tools can favor compounds on one over the other method. This can limit the analytical coverage of compounds. Therefore, it is important to use complementary tools in suspect screening, not just considering chromatography, but also in terms of software and libraries.

The highest median molecular mass and polarity (log D at pH 7.4) was observed for compounds identified by LC (Figure 1 A, B). The widest range in molecular mass and polarity was observed for SFC, whereas GCxGC detected in average the lowest molecular weight compounds. As expected, the deri-

vated GC compounds had the highest Henry's law constant ( $k_H$ ) with median values of  $3E^{-8}$ ,  $9E^{-9}$ ,  $3E^{-9}$  for GC, SFC and LC, respectively (Figure 2C). This shows the importance of including GCxGC with derivatization for very polar and semi-volatile compounds, even though the very volatile compounds will be lost because of the sample preparation workflow (evaporation to dryness for derivatization). To our knowledge, this is the first study showing the advantage of using GCxGC with derivatization to expand the detection capabilities for persistent and mobile compounds in wastewater.

The identified CECs were all present in Pubchem, which was used to annotate the use of these compounds (Figure 2E). For LC, >75% of the compounds were annotated to specific use, whereas >90 % of these were related to pharmaceuticals. For SFC and GCxGC, >50 % of the compounds were without annotation. The remaining annotated compounds were allocated to pharmaceutical (83 % SFC and 72 % GCxGC) and industrial use (8 % SFC and 19 % GCxGC). Pesticides/biocides was the highest for LC with 11 compounds and the lowest for SFC with two compounds, which confirms previous analysis, where pesticides showed better retention with the RP-LC method compared to the polar SFC method [7]. With SFC and GCxGC, industrial compounds were also higher represented compared to LC.

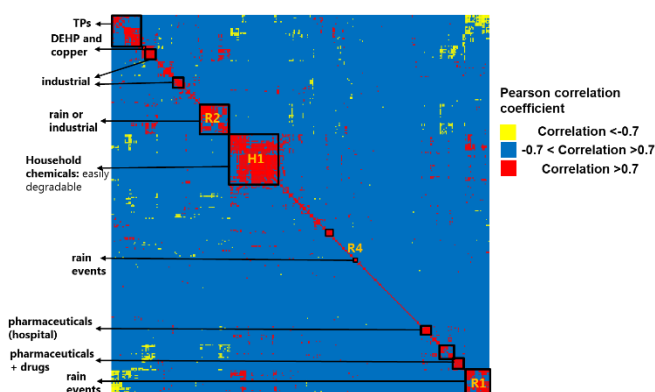


**Figure 1:** Box-plot distribution (10-90% range) of all detected chemicals at the respective platform regarding molecular weight (A), polarity as log D value at pH 7.4 (B) and volatility as Henry law constant ( $k_H$ ) (C). The Venn-Diagram shows the intersection of detected CECs by the respective workflow and (D) shows the annotation of the detected compounds for each of the three methods.

Most of the compounds were detected in both WWTPs. Seven compounds showed >99 % higher peaks in the capital region effluents of WWTP A compared to WWTP B (7,4'-dihydroxyisoflavone, allyl propyl disulfide, 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol, phthalimide, quinine, 6PPD-quinone, naproxen) and six compounds showed peaks >99 % in W-B compared to W-A (paclobutrazol, 1,1-dichloro-2,2-diphenylethane, eugenol, thiachlopid, diphenyl-phosphonic acid, fenhexamid).

## 2.2. Correlation between CECs and meta data

Correlation was established between the CECs and metadata of WWTP A (Figure 2). In the following, the two correlation clusters with most CECs included are explained more in detail.



**Figure 2:** Correlation heatmaps for all variables (336 compounds and metadata) for WWTP A. Clusters are indicated by a black frame.

### 2.2.1. Correlations including household chemicals

The cluster with most variables (H1 in Figure 2) includes chemicals which can be assigned to typical household chemicals, such as caffeine, nicotine, or common pharmaceuticals as atenolol, naproxen, ibuprofen and valsartan. These chemicals are known to degrade easily in WWTPs [8]. The influent flow

( $m^3/h$ ) as variable was also present in cluster H1, showing that the time of the day was a driving factor for the chemical concentration and for the inflow. Flow and chemicals were peaking for samples taken at 8 pm as a 4-hour composite sample and decreasing in intensity for samples taking from 8 am in the morning. Samples highly influenced by rain events (5 to 13 mm within the last 48 h prior to sampling) are coined by an overall higher flow. The compounds from this pattern (H1) follow the flow trend - the higher the flow rate, the higher the concentration of the compounds in effluents, which could be explained by lower hydraulic retention time (HRT): Compounds have less time to degrade, which results in higher concentration in the effluent. Thus, in the investigated WWTP, the overall efficiency is decreasing by rain events. Compounds from household are discharged with higher concentration after a rain event, even though the wastewater flow from households is diluted by rain water.

### 2.2.2. Rain event correlations

CECs in the second biggest clusters were related to rain events (Figure 3, R1 and R2). In the sampling period, three rain events were captured, which were affecting 11 of the 18 samples. The precipitation was directly correlating to 15 CECs in R1. The compounds could be classified to seven pesticides/biocides (6 out of 7 were fungicides), rubber antioxidants (6 PPD and its TP 6PPD-Q), vulcanization accelerators (1,3-diphenylguanidine and benzothiazole-2-sulfonic acid), fire retardant (triphenylphosphate) and industrial or various classes of use (2,4-di-tert-pentylphenol, hexylamine, umbelliferone).

Three compounds were clustering as R2 and showed highest intensities in three samples (15-17) after the highest consecutive rain event in the catchment area. The compounds were hexamethoxymethylmelamine (cross-link agent for rubbers), terbutryn (herbicide used as biocide) and 3,3-diphenylacrylonitrile (industrial).

Rain events significantly impact pollution levels through WWTP discharges, highlighting the challenges in developing effective advanced treatment strategies for rain-influenced wastewater. However, our study also reveals opportunities to track rain-related CEC discharge patterns by establishing correlations.

## 3. Conclusion

In conclusion, we showed that analyzing samples on other platforms than RP-LC-HRMS greatly enhanced the number of CECs detected, with GCxGC -HRMS almost doubling the number of unique compounds detected compared to the RP-LC-HRMS. Many CECs clustered based on their sources and WWTP flow rates, with their occurrence varying significantly over time and being strongly influenced by rain events. Further research is essential to better understand the sources, behavior, and toxicological impacts of emerging contaminants in wastewater systems.

## 4. References

1. Schollée, J. E., Schymanski, E. L., Avak, S. E., Loos, M., & Hollender, J. (2015). Prioritizing unknown transformation products from biologically treated wastewater using high-resolution mass spectrometry, multivariate statistics, and metabolic logic. *Analytical Chemistry*, 87(24), 12121–12129. <https://doi.org/10.1021/acs.analchem.5b02905>
2. Tisler, S., Engler, N., Jørgensen, M. B., Kilpinen, K., Tomasi, G., & Christensen, J. H. (2022). From data to reliable conclusions: Identification and comparison of persistent CECs and transformation products in 37 wastewater samples by non-target screening prioritization. *Water Research*, 219:118599. <https://doi.org/10.1016/j.watres.2022.118599>
3. Neale, P. A., O'Brien, J. W., Glauch, L., König, M., Krauss, M., Mueller, J. F., Tschärke, B., & Escher, B. I. (2020). Wastewater treatment efficacy evaluated with in vitro bioassays. *Water Research X*, 9:100072, <https://doi.org/10.1016/j.wroa.2020.100072>
4. Hollender, J., Schymanski, E. L., Singer, H. P., & Ferguson, P. L. (2017). Nontarget screening with high resolution mass spectrometry in the environment: ready to go? *Environmental Science and Technology*, 51(20), 11505–11512. <https://doi.org/10.1021/acs.est.7b02184>
5. Zahn, D., Neuwald, I. J., & Knepper, T. P. (2020). Analysis of mobile chemicals in the aquatic environment - current capabilities, limitations and future perspectives. *Analytical and Bioanalytical Chemistry*, 412(20), 4763–4784. <https://doi.org/10.1007/s00216-020-02520-z>
6. Hollender, J., Schymanski, E. L., Ahrens, L., Alygizakis, N., Béen, F., Bijlsma, L., Brunner, A. M., Celma, A., Fildier, A., Fu, Q., Gago-Ferrero, P., Gil-Solsona, R., Haglund, P., Hansen, M., Kaserzon, S., Kruve, A., Lamoree, M., Margoum, C., Meijer, J., Krauss, M. (2023). NORMAN guidance on suspect and non-target screening in environmental monitoring. *Environmental Sciences Europe*, 35(1), 75. <https://doi.org/10.1186/s12302-023-00779-4>
7. Lacina P., Mravcová L., Vávrová M (2013). Application of comprehensive two-dimensional gas chromatography with mass spectrometric detection for the analysis of selected drug residues in wastewater and surface water, *Journal of Environmental Sciences*, 25 (1). [https://doi.org/10.1016/S1001-0742\(12\)60006-0](https://doi.org/10.1016/S1001-0742(12)60006-0)
8. Kilpinen, K., Devers, J., Castro, M., Tisler S., Jørgensen M., Christensen, Jan H. (2023): Catchment area, fate, and environmental risks investigation of micropollutants in Danish wastewater. *Environmental Science and Pollution Research* 30, 121107–121123 (2023). <https://doi.org/10.1007/s11356-023-30331-z>
9. Tisler, S., Savvidou, P., Jørgensen, M. B., Castro, M., & Christensen, J. H. (2023). Supercritical fluid chromatography coupled to high-resolution mass spectrometry reveals persistent mobile organic compounds with unknown toxicity in wastewater effluents. *Environmental Science & Technology*. 57(25), 9287–9297. <https://doi.org/10.1021/acs.est.3c00120>
10. Margot, J., Rossi, L., Barry, D. A., & Holliger, C. (2015). A review of the fate of CECs in wastewater treatment plants. *Wiley Interdisciplinary Reviews: Water*, 2(5), 457–487. <https://doi.org/10.1002/wat2.1090>

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