SOLID-PHASE EXTRACTION AS PROMISING SAMPLE PREPARATION METHOD FOR COMPOUNDS OF EMERGING CONCERNS ANALYSIS

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ABSTRACT

Today's environment is under the influence of numerous substances and most of them are not covered by current national and international regulations. Substances that are classified as contaminants of emerging concern, CECs, are not regulated nor systematically controlled in the environment, and they belong to the different chemical classes such as pesticides in current use, pharmaceutically active compounds (PhACs), personal care products, illicit drugs, hormones, micro- and nano-plastics, per- and poly-fluoroalkyl substances (PFAS), and many others. Once released CECs end up in surface water where they can be either accumulated or transported to the other environmental compartments, i.e. soil (by irrigation), underground water, or drinking water. The aim of the research was to investigate the efficiency of different selected solid-phase extraction (SPE) cartridges for preparation of water samples for simultaneous analysis of several CECs. For this purpose, a model mix solution of selected CECs in concentration relevant to environmental appearance was used. The selected CECs (14 PhACs, 11 pesticides in current use, and 4 PFAS) represented emerging contaminants with different properties, including polarities, and varying adverse effect on the environment and human health. Different types of sorbents were investigated: (*i*) commercial single-layer HLB, (*ii*) homemade multi-layer I: HLB plus a mix of WAX, WCX, and PPL, and (*iii*) multi-layer II: mix of WAX, WCX, and PPL plus HLB. The results revealed that the single-layer sorbent showed better efficiency in extraction of the analyzed CECs. Multi-layer sorbent should be further investigated to elucidate the possible reason for the poor extraction of some compounds, as development of a balanced extraction for a wider range of contaminants with different polarities, especially for non-target analysis of chemical residues, is required in order to capture the occurrence of the full profile of micropollutants.

Keywords: compounds of emerging concerns, solid-phase extraction, mass spectrometry, pharmaceuticals, pesticides in current use, per- and poly-fluoroalkyl substances

1. INTRODUCTION

One of the most important resources for sustaining life on Earth is water and access to clean and safe freshwater is a major challenge for humanity. This is a consequence of the increase in population, rapid urbanization, harmonization of human needs with agricultural and other activities and productions, and climate change [1, 2]. Chemical substances that are present in water resources include many different types of compounds that have been shown or are suspected to have adverse effects on human health, collectively known as contaminants of emerging concern - CECs [3]. These substances are considered "emerging" as results of the ongoing studies and the latest discoveries. The term "contaminants of emerging concern" does not always refer to recently approved new chemicals that just recently (unintentionally, intentionally, or accidentally) reached the environment, but can also be used to describe specific molecules that have been present in the environment for a long time, but they have been just recently detected because of the application of the latest advanced instrumental analysis or because new information appeared revealing their risks and biological impacts [4]. Some of the compounds included in that large group are: artificial sweeteners, algal toxins, biocides, disinfection by-products, drugs of abuse, emerging brominated flame retardants, hormones and other endocrine disrupting compounds, nanomaterials, microplastics, organophosphate flame retardants and plasticizers, perfluoroalkyl and polyfluoroalkyl substances, personal care products, polar pesticides and their degradation/transformation products and siloxanes [5]. CECs can enter the environment from a variety of anthropogenic activities such as agriculture, households, mining, industrial, and health work, but wastewater treatment plants are their main source. When pollutants are

released into the environment in high concentrations over small areas, such sources are referred to as point sources (e.g., industrial activities or sewage treatment plants), and when they are released in low concentrations over large areas, they are referred to as diffuse (e.g., rain overflow in urban or industrial areas) [6]. After reaching the water resources, further transport of CECs through the environmental compartments depends on the physical and chemical properties of the pollutants and the conditions of the environment in which they are found [7]. These contaminants typically exist at low concentrations in the environment (in the range of pg/L to mg/L), necessitating further research into the possibility of unintended physiological harm to both people and wildlife [4]. Tandem mass spectrometry (MS-MS) in combination with liquid or gas chromatography represents the method of choice for CECs analysis due to the specificity and sensitivity and consequent undoubted identification and quantification with very low method detection limits [8]. However, in order to remove the possible interfering substances from the samples that can hinder the detection at low levels, and to concentrate the target analytes often occurring at low levels, it is necessary to adequately prepare the sample for targeted MS/MS analysis. For the CECs extraction and efficient reduction of interferences and matrix effects, solid-phase extraction (SPE) is the most widely used technique [9]. The choice of sorbent packed in different SPE holders depends on the polarity of the targeted analytes. On the market, there are numerous SPE sorbents produced to enable efficient clean-up of samples for subsequent analysis of different classes of CECs. Because of wide varieties of the CECs' physicochemical properties ((e.g., molecular weight, dipole moment, polarizability, Van der Waals volume, surface area, acidic or basic character in solution, octanol/water partition coefficient, solubility, etc.) there is no single SPE product capable to efficiently retain/extract all the compounds. This is the limitation in the case of the suspect and non-target screening analysis by high-resolution mass spectrometry when the extraction should be performed in a way to extract as wide as possible range of contaminants with varying polarities, enabling capturing the full profile of micropollutants. As a result, the optimization of the sample preparation step is crucial during the analysis of CECs. Recently, the usage of more than one SPE sorbent in such cases is a general trend in the field of CECs extraction from different environmental matrices. The purpose of this research was to evaluate the extraction efficiency for selected CECs (PhACs, pesticides in current use, and PFAS) on single-layer and homemade multi-layer SPE sorbents, selected because of their known ability to efficiently retain polar and non-polar compounds (HLB), strong acidic compounds (WAX), strong basic compounds (WCX), and even the most polar classes of analytes (PPL).

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All the solvents used in this study were of LC-MS or HPLC grade. Methanol (MeOH) and ethyl acetate (EtOAc) were provided by VWR International (Radnor, USA), and formic acid (10%, LC-MS grade) was obtained from J.T. Baker (Deventer, The Netherlands). Ammonia (25-weight % solution) was purchased from Chem lab (Zedelgem, Belgium). Nitrogen (99.995%) used to enhance the evaporation of organic solvents after the SPE elution was purchased from Messer Tehnogas A.D. (Belgrade, Serbia). A Mili-Q-Advantage system from Millipore (Molsheim, France) was used to obtain high-performance liquid chromatography (HPLC) -grade water. The cartridges used for SPE were Oasis HLB (200 mg, 6 mL), WAX (150 mg, 6 mL), and WCX (150 mg, 60 µm particle size, 6 mL) obtained from Waters Corporation (Milford, MA, USA). Bond Elut -PPL (500 mg, 63–150 µm particle size, 6 mL) sorbent was purchased from Agilent technologies (Santa Clara, CA). The analytes included in this study are CECs of great interest to the environment, i.e. PhACs, pesticides in current use, and PFAS (Tab. 1). Selection of compounds to be included in the list was based on their environmental occurrence, relevance for the EU Water Framework Directive and its latest updates (e.g. proposal for revision of water legislation), and previous group experience [10][11][12]. The list of selected compounds accounts for 29 CECs with a wide range of physicochemical properties (Tab. 1). Tab. 1 summarizes the names of target compounds, CAS numbers, molecular formulas, molecular masses, octanol-water partition coefficients in a form of Log P, and classes of compounds with

respect to their application. Analytical reference standards were obtained from Sigma Aldrich (St. Louis, MO, USA) or LGC (Augsburg, Germany) and were of high purity grade.

Table 1. General information regarding the target compounds included in this study

Compound name	CAS number[1]	Molecular Formulae[1]	Monoisotopic mass g/mol[1]	Log P[1]	Classification[1]
Acetamiprid	135410-20-7	$C_{10}H_{11}ClN_4$	222.067	1.51	insecticide
Carbaryl	63-25-2	$C_{12}H_{11}NO_2$	201.079	2.36	insecticide
Carbofuran	1563-66-2	$C_{12}H_{15}NO_3$	221.105	1.97	insecticide
Dimethoate	60-51-5	$C_5H_{12}NO_3PS_2$	229.000	0.78	insecticide
Linuron	330-55-2	$C_9H_{10}Cl_2N_2O_2$	248.012	3.20	herbicide
Malathion	121-75-5	$C_{10}H_{19}O_6PS_2$	330.036	2.36	insecticide
Methidathion	950-37-8	$C_6H_{11}N_2O_4PS_3$	301.962	2.20	pesticides
Omethoate	1113-02-6	$C_5H_{12}NO_4PS$	213.022	-0.74	insecticide
Phosphamidon	13171-21-6	$C_{10}H_{19}ClNO_5P$	299.069	0.79	insecticide
Propiconazole	60207-90-1	$C_{15}H_{17}Cl_2N_3O_2$	341.070	3.72	fungicide
Tebuconazole	107534-96-3	$C_{16}H_{22}ClN_3O$	307.145	3.70	insecticide
Acetaminophen	103-90-2	$C_8H_9NO_2$	151.063	0.46	analgesic
Atenolol	29122-68-7	$C_{14}H_{22}N_2O_3$	266.163	0.16	cardio selective beta-blocker
Clarithromycin	81103-11-9	$C_{38}H_{69}NO_{13}$	747.477	3.16	macrolide antibiotic
Diclofenac acid	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	295.017	4.51	nonsteroidal anti-inflammatory drug
Diltiazem	42399-41-7	$C_{22}H_{26}N_2O_4S$	414.161	2.8	calcium channel blocker
Erythromycin	114-07-8	$C_{37}H_{67}NO_{13}$	733.461	2.6	macrolide
Furosemide	54-31-9	$C_{12}H_{11}ClN_2O_5S$	330.008	2.03	diuretic
HCTZ (hydrochlorothiazide)	58-93-5	$C_7H_8ClN_3O_4S_2$	296.964	-0.07	diuretic
Losartan	114798-26-4	$C_{22}H_{23}ClN_6O$	422.162	1.19	angiotensin 2 receptor blocker
Propranolol	525-66-6	$C_{16}H_{21}NO_2$	259.157	3.48	beta-adrenergic blocker
Sotalol	3930-20-9	$C_{12}H_{20}N_2O_3S$	272.119	1.1	antiarrhythmic
Salbutamol	18559-94-9	$C_{13}H_{21}NO_3$	239.152	1.4	beta2-adrenergic agonist
Bezafibrate	41859-67-0	$C_{19}H_{20}ClNO_4$	361.108	3.74	agonist of peroxisome proliferator
Carbamazepine	298-46-4	$C_{15}H_{12}N_2O$	236.095	2.77	mood Stabilizer
PFHxA[2]	307-24-4	$C_6HF_{11}O_2$	313.980	4.24	industrial chemical
PFHpA[3]	375-85-9	$C_7HF_{13}O_2$	363.977	2.05	industrial chemical
PFOA[4]	335-67-1	$C_8HF_{15}O_2$	413.974	5.48	industrial chemical
PFNA[5]	375-95-1	$C_9HF_{17}O_2$	463.970	6.26	industrial chemical

[1]PubChem (https://pubchem.ncbi.nlm.nih.gov/), [2]PFHxA - Perfluorohexanoic acid, [3]PFHpA - Perfluoroheptanoic acid, [4]PFOA - Perfluorooctanoic acid, [5]PFNA - perfluorononanoic acid

2.2. Sample preparation

The aliquots of HPLC-grade water (200 mL) were spiked with selected CECs to obtain the final concentrations of each compound of interest to be 300 ng/L. Three different SPE sorbents were evaluated in this study – one single and two multi-layer (Fig. 1). Oasis HLB cartridge (200 mg, 6 ml), with no modification, was used as a single-layer sorbent, while as multi-layer sorbents two combinations of sorbents were investigated: (*i*) multi-layer I with upper layer containing 200 mg of Oasis HLB sorbent and bottom layer containing a mixture of 150 mg of Bond Elut PPL, 100 mg of WAX and 100 mg of WCX and (*ii*) multi-layer II with upper layer containing mixture of 150 mg of Bond Elut PPL, 100 mg of WAX and 100 mg of WCX, and bottom layer containing 200 mg of Oasis HLB sorbent. The cartridges were positioned in

a vacuum manifold and conditioned by passing 2 x 5 mL of MeOH and 2 x 5 mL of HPLC water through them. Then, the samples (200 mL) were loaded on SPE cartridges, and the flow was settled up at one to two drops on each three to four seconds for the maximum retention of the compounds. When the total volume of each sample was passed, the cartridges were dried to remove excess of water by passing air through them for 3 hours. Single layer cartridges were eluted with two portions of 4 mL MeOH. Multi-layer cartridges were eluted with 2 x 3 mL 5% NH_3 in MeOH:EtOAc (50:50 v/v), followed by 2 x 3 mL of 2% formic acid in MeOH:EtOAc (50:50 v/v) and finally with 3 mL of MeOH:EtOAc (50:50 v/v). The eluates were then evaporated to dryness at 30 °C under a gently nitrogen stream and reconstituted in 1 mL of first gradient of mobile phase. The multi-layer method was previously used for analysis of wastewater-borne pollutants in surface water [13], while single-layer SPE was applied for PFAS analysis in surface and wastewater samples [12].
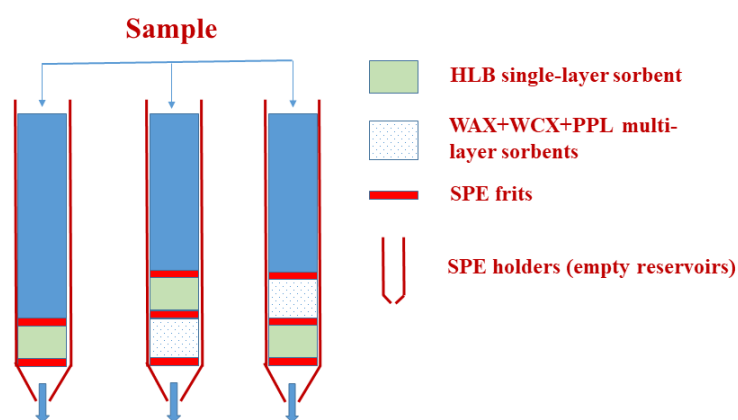

Figure 1. Designs of experiments for solid-phase extraction optimization

2.3. Instrumental analysis

Instrumental analysis of CECs was done by high performance liquid chromatography coupled to triple quadrupole mass spectrometry, UHPLC–MS/MS (Thermo Fisher Scientific, USA). Hypersil GOLD™, 50 × 2.1 mm i.d., 1.9 µm column (Thermo Fisher Scientific, USA) was used with a flow rate of 400 µL/min, and the column temperature was maintained at 30 ºC. The injection volume was 10 µL. The mobile phase consisted of eluent A containing water/formic acid (99.9:0.1, v/v) and 5 mM ammonium acetate, and eluent B, which consists only of MeOH. The gradient program started with 5 % of eluent B, increasing to 95% in 4.5 min, raising to 100% in the following 0.1 min and held 1.9 min and then back to initial conations. Total time of the run was 10 min. For analytes detection, triple quadrupole mass spectrometer (MS/MS) TSQ Vantage (Thermo Fisher Scientific, USA) equipped with heated-electrospray ionization probe (HESI-II, Thermo Scientific, USA) was used. Parameters of the ion source were as follows: spray voltage - 3.4 kV, vaporizer temperature - 250°C, sheath gas pressure - 40 arbitrary units, auxiliary gas pressure - 10 arbitrary units, and capillary temperature - 270°C. During the optimization of mass spectrometric conditions, each analyte of interest in concertation of 1 µg/mL was directly infused in the MS to optimize the parameters such as parent and product masses, collision energies, polarity, etc.

2.4. Quality control

Calibration curves were generated using linear regression analysis. The calibration was accepted if the $R^2>0.9900$ over the established concentration range from 1 to 400 ng/L. The calibration solutions were

prepared in the first gradient phase used for chromatographic separation (H_2O: MeOH = 95:5.0, v/v). Stock solutions of CECs were prepared in MeOH. During the preparation of calibration solutions, an equal amount of stock solution and solution of the first gradient of the mobile phase were used (50 µL + 950 µL, respectively) in order not to disturb the equilibrium of the UHPLC gradient program at the beginning of the chromatographic run. The method detection limits (MDLs) and method quantification limits (MQLs) were determined for analysed compounds corresponding to the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. Recovery experiments were done by spiking the HPLC-grade water sample. Precision of the method was determined by calculating the relative standard deviation (%RSD) of the triplicate spiked samples.

3. RESULTS AND DISCUSSION

Mass spectrometric parameters, i.e. CECs parent masses, product masses, collision energies, and the time windows in the chromatographic run where the particular CEC eluates from the column, were optimized for each studied compound (Tab. 2). The total chromatographic run lasted 10 minutes and the compounds of interest were eluted from the column in the first five minutes of the chromatographic run. The most polar compounds were eluted first during the UHPLC chromatographic separation and higher MQL were observed for these compounds in comparison with the compounds eluted later (Fig. 2).

Table 2. Mass spectrometric parameters of compounds of interest

| Name	Retention time, min	Parent mass, m/z	Product mass 1/ Collision energy (eV)	Product mass 2/ Collision energy (eV)	Start time, min	Stop time, min	S-lens, V	Polarity
Sotalol	1.09	273.140	133.080/27	255.070/11	0.60	2.10	74	+
Acetaminophen	1.18	152.100	93.100/22	110.100/16	0.20	2.50	71	+
HCTZ	1.18	295.950	204.940/24	268.880/20	0.50	2.30	96	-
Omethoat	1.19	213.981	124.970/25	182.990/11	0.50	2.00	69	+
Salbutamol	1.26	240.160	148.070/18	222.110/10	0.70	2.20	69	+
Atenolol	1.30	267.200	145.060/26	190.060/18	0.70	2.20	93	+
Dimethoate	2.55	229.993	125.000/23	198.950/10	2.00	3.00	64	+
Acetamipirid	2.70	223.074	73.020/53	126.000/21	2.00	3.50	82	+
Phosphamidon	3.21	301.065	127.010/23	227.970/12	2.00	4.00	88	+
Furosemide	3.23	328.990	204.940/23	284.950/17	2.00	4.00	91	-
Propanolol	3.32	260.184	155.130/25	183.060/17	2.90	4.00	87	+
Carbofuran	3.38	222.100	123.060/22	165.110/12	3.00	4.50	67	+
Carbaryl	3.53	202.100	127.050/32	145.080/11	3.00	4.50	46	+
Diltiazem	3.55	415.100	149.990/40	177.960/25	3.00	4.60	115	+
Carbamazepin	3.61	237.100	192.100/24	194.100/19	3.00	4.20	92	+
Erithromycin	3.77	734.300	157.970/30	576.180/18	3.00	5.00	147	+
PFHxA	3.82	312.820	119.030/24	269.040/11	3.40	4.20	51	-
Methidation	3.90	302.972	85.010/19	145.090/7	1.00	5.00	60	+
Losartan	3.92	420.982	126.900/35	179.100/26	3.00	5.00	93	-
Clarithromycin	4.06	748.300	157.960/28	590.210/17	3.00	5.00	149	+
Linuron	4.08	249.031	159.960/18	182.020/16	1.00	5.00	81	+
Benzofibrate	4.10	360.100	153.970/30	273.990/20	3.20	5.60	90	-
PFHpA	4.15	362.940	169.110/19	319.110/10	3.800	4.60	80	-
Malathion	4.20	331.000	124.990/23	284.940/27	3.00	5.00	65	+
PFOA	4.44	412.942	219.100/20	369.070/12	4.00	4.90	80	-
Diclofenac acid	4.54	294.000	249.960/14	-	4.00	5.50	65	-
Tebucanazol	4.60	308.100	70.100/21	125.000/36	1.00	6.00	96	+
Propiconazole	4.64	343.058	158.930/34	160.000/33	4.00	5.00	99	+
PFNA	4.66	462.944	219.020/17	419.010/17	4.20	5.10	64	-
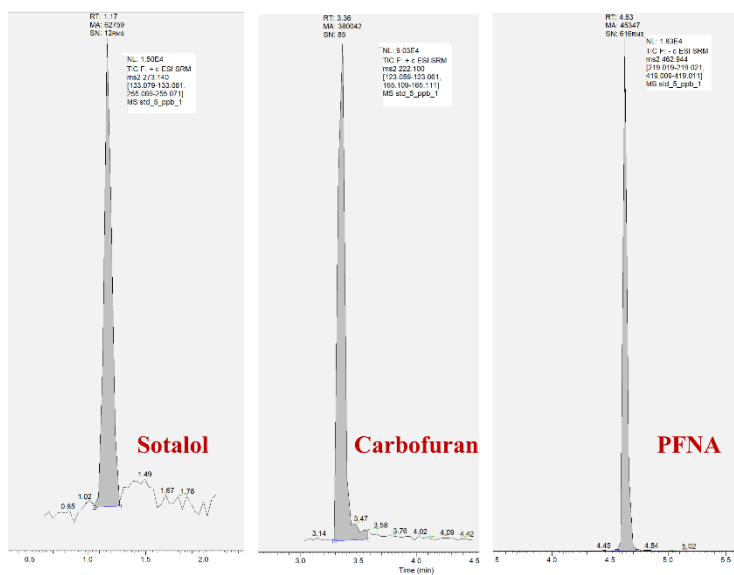
Figure 2. Chromatographic peaks of sotalol, carbofuran, and PFNA at a concentration of 5 ng/mL

Regarding the recovery experiments, as it was stated ultra-pure water spiked with the analytes of interest in environmentally relevant concentration was used for the in-house validation of the method. In total, 29 CECs (14 PhACs, 11 pesticides, and 4 PFAS) were selected for this study. The analytes differ regarding the physicochemical properties. The method was validated to final CECs concentration in samples of 300 ng/L, in triplicate, covering the typical environmental concentrations of CECs in surface water. The in-house validation parameters included accuracy (recovery rates), precision (% RSD), linearity (R^2), MDL, and MQL. All the compounds showed acceptable linearity ($R^2 > 0.9900$). The procedure of calibration solutions preparation, as previously explained, is essential for obtaining a good correlation among the calibration points covering the range from MQL to the maximum expected concentration in the environmental samples. Chromatographic separation and ESI ionization are dependent on many parameters including the composition of solvents in which samples are dissolved. Additionally, the presence of salts and acids (e.g., ammonium acetate and formic acid) influences the abundance of analytes during the ESI ionization. As the analytes are dissolved in the first gradient of the mobile phase (95% of A phase vs. 5% of B phase), the preparation of calibration solution in the same matrix was a very important decision. During the preparation of particular calibration concentration, the ratio between the A and B phases is not significantly disturbed as only 50 µL of working standard solution was used for the preparation of the desired calibration concentration. Method quantification limits were in the range from 0.20 to 10 ng/L (for the majority of the analytes MQLs were lower than 1.0 ng/L), which are reliable results for the application of the method for CECs quantification in environmental matrices. MDLs and MQL are comparable to those reported in similar studies [13, 14]. In Fig. 3 are shown recovery results (RSD<20%) for single and multi-layer experiments. As can be observed HLB sorbent showed slightly better retention for the majority of CECs, particularly pesticides. Multi-layer sorbents contained the same amount of HLB sorbent plus the sorbents capable to retain of strong acidic compounds (WAX) and strong basic compounds (WCX) as well as PPL sorbent designed to retain even the most polar classes of analytes. The multi-layer sorbents were prepared in the laboratory by mixing the WAX, WCX, and PPL sorbents and packing them manually. Another difference was the elution solvents. The compounds were eluted from HLB sorbent using the two portions of MeOH (2x4 mL) [12], while the analytes from multi-layer sorbents were eluted using the combination of solvents (MeOH and EtOAc) [13] as was earlier explained in detail. For the most of analyzed CECs, the recoveries were in the range of 60-110%. It

was expected that some compounds demonstrate lower recoveries. The lower efficiency of extraction was observed for malathion, furosemide, diltiazem, and losartan. Similar results for losartan were observed in the study of Gómez-Navarro et al. (2023) [13], where the same combination of SPE sorbents and elutants composition were used. Higher recovery values were observed for diltiazem and furosemide than in this study. In the mentioned study recovery experiments were investigated at significantly higher levels (5, 50, and 500 µg/L), while in this study recovery experiments were done at the spiking level of 0.300 µg/L.

On the market, there are only a few available multi-layer sorbents, whoever, the combination of sorbents used in this study (HLB as the upper or the bottom layer with a mix of WAX, WCX, and PPL) as a commercial product is not available on the market. One of the reasons for the better retention of selected CECs by hydrophilic-hydrophobic polymer is that the compactness of the sorbent bed is not violated. On the other hand, the multi-layer sorbent was prepared in the laboratory and the compactness of the sorbent bed is violated. The sorbent type of HLB cartridges (copolymer) enables retention of compounds with a wide polarity range and do not have pH limitation. However, hydrophilic/hydrophobic polymeric material fails in recovering ionic compounds and the short-chain compounds e.g., some emerging PFASs, which are better recovered using anion exchange sorbents [14]. As the aim of this study was to optimize the method for efficient extraction of as much as possible CECs from model solution for further application in untargeted analysis of contaminants in river water samples further investigations will be directed to the optimization of multi-layer sorbent.
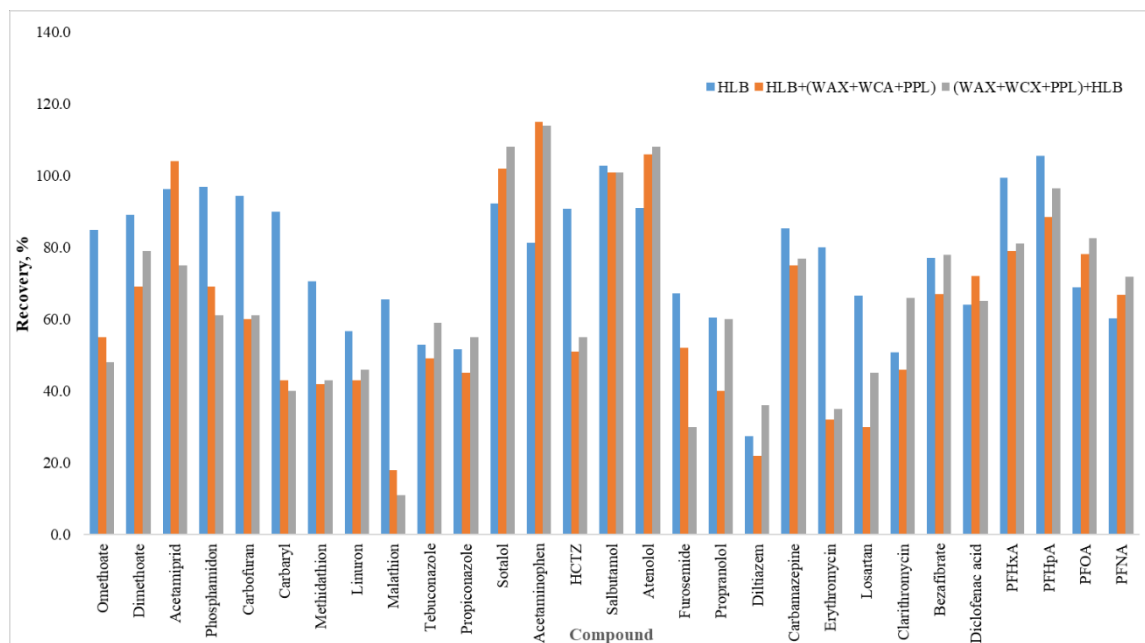

Figure 3. Recoveries experiments for single and multi-layer experiments

4. CONCLUSIONS

The use of different sorbents demonstrates different efficiencies and a few challenges. The use of single-layer SPE sorbent based on hydrophilic-lipophilic balance (HLB) demonstrated better retention of selected CECs (14 PhACs, 11 pesticide in current use, and 4 PFCs) in comparison to multi-layer sorbent. Although multi-layer sorbent contained also a layer of HLB together with other sorbents developed to retain strong acidic compounds, strong basic compounds, and some of the most polar classes of analytes, its in-house

preparation and usage of complex elution solvents demonstrated lower efficiency of retention in comparison to the commercial HLB solid-phase extraction. The poor extraction of some CECs when multi-layer sorbent was used, should be further investigated to elucidate the possible reason (e.g., insufficiently optimized solvent composition for the elution and/or inhomogeneity of the mixed sorbent bed). The possibility to extract as widest possible range of CECs is pre-request for the novel suspect and non-target screening approaches by HRMS that can capture the occurrence of the full profile of micropollutants, indicating more complete environmental chemical status compared to targeted MS/MS analysis.

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