analytical chemistry

Article

pubs.acs.org/ac

Rapid Screening of Acetylcholinesterase Inhibitors by Effect-Directed Analysis Using LC × LC Fractionation, a High Throughput in Vitro Assay, and Parallel Identification by Time of Flight Mass Spectrometry

Xiyu Ouyang,*[,†] Pim E. G. Leonards,[†] Zuzana Tousova,[‡,§] Jaroslav Slobodnik,[‡] Jacob de Boer,[†] and Marja H. Lamoree[†]

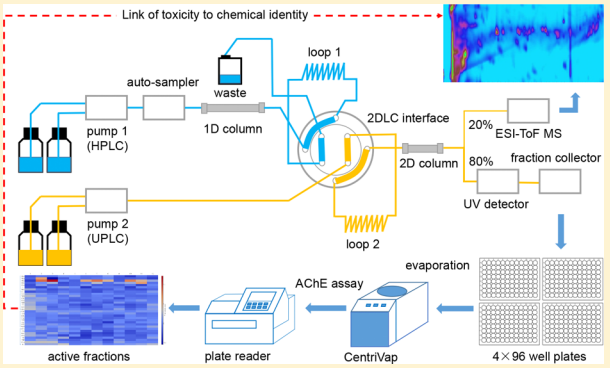
[†]Institute for Environmental Studies (IVM), VU University Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

[‡]Environmental Institute (EI), Okruzna 784/42, 972 41 Kos, Slovak Republic

[§]Faculty of Science, Masaryk University, RECETOX, Kamenice 753/5, 625 00 Brno, Czech Republic

Supporting Information

ABSTRACT: Effect-directed analysis (EDA) is a useful tool to identify bioactive compounds in complex samples. However, identification in EDA is usually challenging, mainly due to limited separation power of the liquid chromatography based fractionation. In this study, comprehensive two-dimensional liquid chromatography (LC × LC) based microfractionation combined with parallel high resolution time of flight (HR-ToF) mass spectrometric detection and a high throughput acetylcholinesterase (AChE) assay was developed. The LC × LC fractionation method was validated using analytical standards and a C18 and pentafluorophenyl (PFP) stationary phase combination was selected for the two-dimensional separation and fractionation in four 96-well plates. The method was successfully applied to identify AChE inhibitors in a wastewater treatment plant (WWTP) effluent. Good orthogonality (>0.9) separation was achieved and three AChE inhibitors (tiapride, amisulpride, and lamotrigine), used as antipsychotic medicines, were identified and confirmed by two-dimensional retention alignment as well as their AChE inhibition activity.



One of the major challenges nowadays in the field of environmental analysis is sample complexity. The huge number of compounds present in the environment due to cumulative human activities is increasing. A significant part of these compounds is believed to have a potential to cause adverse effects on the ecosystem. Besides, complementing extensive programs in many countries that monitor target compounds to protect environmental quality, there is a need to develop, refine, and apply integrated analytical and effect-based tools in a so-called effect-directed analysis (EDA) approach.[1] Conventionally, after sample extraction EDA includes a fractionation based on high performance liquid chromatography (HPLC) where usually dozens of fractions are collected, followed by a biological screening of the fractions by a specific bioassay and analysis by either liquid or gas chromatography (LC or GC) coupled to high resolution mass spectrometry (HRMS) of the active fractions to identify the compounds responsible for the observed effects. By applying different bioassays covering a broad range of toxicological end points, it was proven that EDA is an important tool for environmental quality assessment to identify contaminants having a toxicological effect.[2−4] Yet, when dealing with an extremely complex sample matrix such as WWTP (wastewater treatment plant) effluent, sediment, or biological tissue, it usually happens that still tens or even hundreds of MS peaks are detected in each of the active fractions, usually due to limited separation power provided in the fractionation procedure. Straightforward identification of bioactive chemicals in these active fractions therefore still remains challenging unless two or more additional fractionation steps would be performed.[2,4,5]

Furthermore, as environmental contaminants originate from a variety of sources such as industrial activities, agricultural production or urban sewage, their physicochemical properties also significantly differ. The persistent organic pollutants (POPs) including polycyclic aromatic hydrocarbons (PAHs) and dioxin-like compounds are usually nonpolar or very weakly polar compounds. Pesticides are usually more polar than POPs, while some of the pharmaceuticals and personal care products

Received: November 13, 2015
Accepted: January 12, 2016
Published: January 12, 2016

DOI: 10.1021/acs.analchem.5b04311
Anal. Chem. 2016, 88, 2353−2360

(PPCPs) and their corresponding metabolites emerging in the environment may have a strongly polar character. Therefore, one single HPLC separation mechanism for sample fractionation may not be sufficient to fully address the chemical properties of complex environmental samples.

To further simplify the complexity of the LC fractions, developing and applying an innovative comprehensive analytical tool capable of delivering higher resolution and multiselective fractionation in EDA is required. LC × LC is an emerging technique where an extra, independent LC separation is implemented after the first conventional separation to achieve greater peak capacity. Theoretically, the peak capacity of LC × LC is the product of the peak capacities of the individual dimensions, provided that the two separation mechanisms are truly orthogonal.[6] Furthermore, stationary phase combinations of the two dimensions in LC × LC may provide multiselective separation of the contents of a sample. In recent years, LC × LC has been successfully applied to complex sample matrices in diverse fields such as proteomics, pharmaceuticals, food and polymer science, etc.[7−10] The application of LC × LC in environmental analysis is also promising; however, there are only very few studies on the use of LC × LC in this field.[11,12] In comparison with GC based techniques, another major advantage of LC × LC in EDA is the straightforward applicability for fractionation. By implementing a post column fraction collector, direct microfractionation in 96 or 384 well plates after LC × LC separation can be easily achieved. In addition, the high resolution fractionation in microplates enables high throughput screening of bioactivity.

Acetylcholinesterase (AChE) inhibitors are chemicals that inhibit the AChE enzyme that hydrolyses the neurotransmitter acetylcholine (ACh) to choline, which may cause accumulation of ACh in the synaptic cleft and result in overstimulation of cholinergic receptors.[13] AChE inhibitors such as organophosphates and carbamates have been widely used as insecticides. They kill insects by inhibiting the AChE enzyme in their nervous system.[14] Some carbamates have also been applied to attenuate presynaptic cholinergic deficits related to Alzheimer's disease.[15] In this study, the AChE inhibition was investigated in LC × LC fractions of WWTP effluents using the classic Ellman's method with modifications.[16,17]

Hyphenation of LC × LC to a high resolution mass spectrometer (HRMS) was established for identification of environmental contaminants. Of all MS detectors, time of flight (ToF) MS is considered to be the most suitable to couple with LC × LC due to its fast scan rate and moderate to high resolving power. However, the high flow rate commonly used in the second dimension of LC × LC is not optimal for most of the ionization sources. By applying a postcolumn splitter, a small portion of the eluent was introduced to the MS while the rest was collected in 96 or 384 well plates using a fraction collector. This parallel approach not only provides an optimal flow rate for the MS interface but also enables a direct correlation of activity in the wells with MS identification. For identification of active chemicals, the accurate mass and isotopic pattern were first evaluated to determine the molecular formula. Then the molecular formulas were searched in online databases for likely candidates, allowing narrow mass errors and isotopic pattern fits. Eventually the tentatively identified compounds were confirmed by two-dimensional retention alignment and bioactivity in the AChE inhibition assay.[11]

EXPERIMENTAL SECTION

Chemicals. Acetonitrile was HPLC grade supplied by Sigma-Aldrich (Zwijndrecht, The Netherlands). Water was obtained from a Milli-Q Reference A+ purification system (Millipore, Bedford, MA, USA). Formic acid and the reagents used in the AChE inhibition assay were purchased from Sigma-Fluka (Zwijndrecht, The Netherlands). The EPA 531.1 Carbamate Pesticide Calibration Mixture was purchased from Restek (Bellefonte, PA, USA). The standards of tiapride, amisulpride and lamotrigine were purchased from Sigma-Fluka. All standards were diluted in 50:50 acetonitrile/water (v/v) to 1−10 μg/mL prior to injection.

Sampling Site. The WWTP in Brno-Modřice, Czech Republic (49.12447N, 16.62697E), serves the urban agglomeration of Brno with a population of over 400,000. The WWTP collects mainly municipal wastewater (WW). However, WW from several industries, hospitals or street runoff contributes to the overall load of emerging contaminants because the sewage system does not differentiate between these sources. The WWTP was modernized in 2004 and equipped with tertiary treatment including nitrogen and phosphorus removal.

WWTP Effluent Sampling and Sample Preparation. The WWTP effluent sample was collected in mid-August 2013 using a large volume solid phase extraction (LVSPE) device (UFZ, Leipzig, Germany; Maxx GmbH company, Rangendingen, Germany).[18] The device enabled extraction of 50 L of water within 4 h and primary on-site fractionation into 3 fractions based on the affinity of different sorbents for distinct compound groups. Water entering the device was filtered with a glass fiber filter (0.63 μm, Sartopure GF+ MidiCap, Sartorius AG, Göttingen, Germany) and then pressurized through three sorbent cartridges mounted in sequence. The first cartridge containing the neutral sorbent polystyrene-divinylbenzene copolymer (PS-DVB; Chromabond HR-X, Macherey Nagel, Düren, Germany −8 g) to capture neutral and semipolar compounds was followed by the second cartridge with a weak anionic exchanger based on PS-DVB sorbent (Chromabond HR-XAW − 3.5 g) to capture acidic compounds. The third cartridge with a weak cationic exchanger also based on PS-DVB sorbent (Chromabond HR-XCW − 3.5 g) capture basic compounds that are cationic at a water pH of 6−8.

Prior to the field sampling, all equipment parts were cleaned with methanol (MeOH) and the sorbent cartridges were preconditioned with a mixture of MeOH and Milli-Q water. In the field, the weather conditions as well as the main physicochemical parameters of the sampled water (temperature, pH, dissolved oxygen, conductivity) were recorded. After the field sampling, the sorbent cartridges were freeze-dried and eluted separately with different solvent mixtures (neutral cartridge: MeOH and ethyl acetate, 1:1, v/v; anionic cartridge: 2% v/v 7N ammonia in MeOH; cationic cartridge: 1.7% v/v formic acid in MeOH). The eluates were filtered through glass microfiber filters (Whatman GF/F: 0.7 μm, VWR, Vienna, Austria) and evaporated to dryness using nitrogen. Each dried eluate was stored at −20 °C until reconstitution in 1 mL of Milli-Q water and acetonitrile (1:1, v/v) for further analysis. In this study only the eluate from the neutral cartridge was investigated. An instrument blank was prepared by extraction of 2 L mineralized LC-grade distilled water according to the procedure used for the field sample.

LC × LC-ToF MS Instrumentation. The LC × LC system integrates an Agilent 1100 auto sampler, an Agilent 1100 HPLC

DOI: 10.1021/acs.analchem.5b04311
Anal. Chem. 2016, 88, 2353−2360

binary pump (first dimension), an Agilent 1290 Infinity UHPLC binary pump (second dimension), and an Agilent 1290 Infinity thermostated column compartment (TCC) with a 2-position/4-port duo valve and two sampling loops (80 μL) installed as the 2D interface (Agilent Technologies, Waldbronn, Germany). The LC × LC module was controlled by the Chemstation version B.04.03 (Agilent Technologies) with 2D-LC add-on. In the first dimension, a ZORBAX Eclipse Plus (1.8 μm, 2.1 × 150 mm ID) C18 Rapid Resolution HD column (Agilent Technologies, Santa Clara, CA, USA) was used and a Phenomenex Kinetex (2.6 μm, 50 × 4.6 mm ID) PFP column (Phenomenex, Torrance, CA, USA) was deployed for the second dimension. After the second column, the flow was split using a QuickSplit adjustable flow splitter (Richmond, CA, USA): 20% was directed to a Bruker micrOTOF time of flight (ToF, resolving power ∼10000) mass spectrometer with an electrospray interface (ESI, Bruker Daltonics, Bremen, Germany) while the remaining 80% went through an Agilent 1260 Infinity variable wavelength detector (VWD) followed by an Agilent 1260 fraction collector (Agilent Technologies). Figure 1 illustrates the configuration of the integrated LC × LC system (valve position 1).

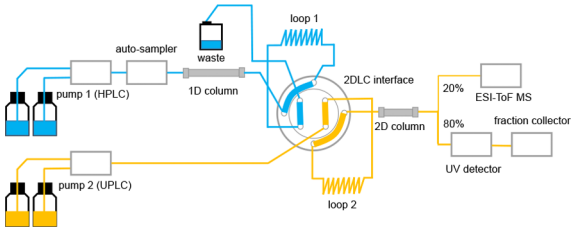


Figure 1. Instrumentation of the developed LC × LC system with parallel ESI-ToF-MS and UV detection followed by fraction collection for EDA.

LC × LC Conditions. The chromatographic conditions of the LC × LC experiments are listed in Table 1 and are the optimized conditions for the carbamate mixture or the WWTP sample.

MS Settings. The start and stop signals of the micrOTOF were initiated by external control via serial ports. The MS data were recorded by Bruker OtofControl 3.0 (Bruker Daltonics), using a scan frequency of 5 Hz in order to collect enough data points for fast separation in the second dimension. The ion source and transfer settings of the MS were adjusted to achieve optimum sensitivity in the selected mass range (50−1000, m/z). The capillary voltage of the ESI was 4500 V with end plate offset −500 V. Due to the relatively high flow rate (400 μL/min) in the second dimension, the nebulizer gas (N2) was operated at 4.0 bar and the drying gas was set to 8 L/min at a temperature of 200 °C. To enable best detection of the most interesting molecules, the capillary exit was set at 100 V with a skimmer voltage of 33.3 V, the hexapole RF was regulated to 90 Vpp and lens 1 prepulse storage was set to 1 μs.

Assessment of Orthogonality. Two different algorithms were applied to evaluation the orthogonality of the separation to characterize the performance of the LC × LC. The normalized surface coverage was first estimated using the surface coverage method described in a previous study.[11] The details of the calculation can be found in SI. The orthogonality was also evaluated by measuring the spread of peaks within the separation space, according to a recent approach.[19]

Table 1. LC × LC Conditions

samples	column combination	injection volume	first dimension LC conditions	second dimension LC conditions
EPA 531.1 carbamate pesticide mixture	C18 × PFP, 25 °C	20 μL	Mobile phase: (A) water; (B) acetonitrile. Gradient: 0 min 20% B, 30 min 45% B, 55 min 80% B, 60 min 80% B. Flow rate: 0.1 mL/min.	Mobile phase: (A) water with 0.1% formic acid; (B) acetonitrile with 0.1% formic acid. Modulation time: 0.6 min. Gradient: 0 min 20% B, shifted to 35% in 25 min and then 80% at 55 min; 0.5 min 40% B, shifted to 85% in 48 min. Flow rate: 2.0 mL/min.
Brno-Modřice WWTP effluent extract	C18 × PFP, 25 °C	20 μL	Mobile phase: (A) water; (B) acetonitrile. Gradient: 0 min 45% B, 35 min 55% B, 55 min 90% B, 60 min 90% B. Flow rate: 0.1 mL/min.	Mobile phase: (A) water with 0.1% formic acid; (B) acetonitrile with 0.1% formic acid. Modulation time: 0.6 min. Gradient: 0 min 45% B, shifted to 50% in 30 min and then 90% at 55 min; 0.5 min 65% B, shifted to 75% in 30 min and then 95% at 40 min. Flow rate: 2.0 mL/min.

DOI: 10.1021/acs.analchem.5b04311
Anal. Chem. 2016, 88, 2353−2360

LC × LC ToF-MS Data Analysis. First, the base peak chromatograms (BPCs) obtained were calibrated by creating a calibration segment before the analysis using the calibration tunemix solution and high precision calibration (HPC) in the instrument software package DataAnalysis version 4.1 (Bruker Daltonics). The calibrated data were then saved as netCDF. Second, the data (both from UV and MS) were evaluated and the two-dimensional counter plots were generated by GC Image 2.3b4 (Lincoln, NE, USA) using the linear interpolation algorithm. Third, tentative compound identifications were carried out using DataAnalysis. The SmartFormula function in DataAnalysis was used to determine possible chemical formulas corresponding to the observed accurate masses and isotopic patterns. Afterward, the CompoundCrawler function was applied to search for known compounds from a large number of databases, such as ChemSpider, NIST and METLIN. The data analysis of the semiquantitative experiments was performed directly in DataAnalysis by integrating the peak areas of a series of concentrations of standards injected.

Fractionation Settings. The fractionation was established after the UV detector by an Agilent 1260 fraction collector. The delay volume (55 μL) was determined according to the Agilent technical note (Part Nr. G1364−90104). For each fractionation, four 96-well microplates (polystyrene F-bottom, Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were placed in the fractionation collector. The total run time (60 min) was evenly divided into 384 (4 × 96) parts, giving 9.375 s collection time for each fraction. The fractionation was performed row by row following the shortest pathway, which is illustrated in the Supporting Information (Figure S1). The needle distance above the wells was 3.5 mm. After the fractionation, volatile solvents were evaporated using a CentriVap concentrator (Labconco, Kansas City, KS, USA) at 40 °C in 6 h, to remove the solvents for the following bioassay screening. Prior to the fractionation process, 10 μL of glycerol solution (10% in water, m/m, Sigma-Fluka) was added to each well as a solvent keeper to enhance the recovery.[20]

AChE Inhibition Assay. The AChE inhibition assay was performed based on Hamers et al.[17] with modifications. The method used *S*-acetylthiocholine-iodide (ATC) as a substrate to evaluate the AChE activity. The ATC hydrolysis was measured by reaction of ATC with 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) to generate the yellow 5-thio-2-nitro-benzoic acid anion, using a UV−vis spectrometer at 412 nm. First, 50 μL of 0.1 M phosphate buffer (KH_2PO_4/K_2HPO_4; pH 7.5) and 50 μL of purified AChE from electric eel (*Electrophorus electricus*; 125 mU/mL in 0.02 M sodium phosphate buffer; pH 7.0) were added to each well of the fractions in the 96-well plates. The plate was incubated for 30 min at room temperature. Then 50 μL of 5 mM DTNB and 50 μL of 0.8 mM ATC were added to each well. Afterward, a kinetic measurement was immediately performed at 412 nm for 5 min (interval 20 s) in a Multiskan FC micro plate photometer (Thermo Scientific, Waltham, MA, USA). For each identified compound (tiapride, amisulpride, and lamotrigine) a dose response curve was determined in triplicate, using a series of 8 concentrations. The dose response curves were plotted and the IC_{50} values were calculated using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Validation of the LC × LC Microfractionation Approach. To evaluate the applicability of LC × LC for microfractionation, a carbamate mixture was fractionated into four 96-well plates followed by the AChE inhibition assay to test the applicability of this workflow before applying it to a real sample. In virtue of the postcolumn flow splitter shown in Figure 1, the LC × LC fractionations were performed in parallel to the ToF-MS analysis, in triplicate. The AChE screening results in the four plates are presented in Figure 2 as a heatmap after comparison with a blank fractionation run. According to the retention times of the compounds in the mixture recorded by the MS, after dead volume correction, it is possible to correlate the observed activity to specific compounds in the mixture. AChE inhibition was observed for each of the fractions known to contain one of the carbamates present in the mixture. Although very good separation was achieved in both dimensions (see Figure S2 in the SI), several compounds caused a response in more than two fractions, which could be attributed to the high concentrations present in the mixture. In addition, the metabolite aldicarb-sulfoxide (produced by cytochrome P450) is roughly 200 times more potent to inhibit AChE than aldicarb itself[21] and therefore led to significant activities in several fractions such as P1D12, P1D11, P1D10, P1C10 and P1C11. The activities in the wells P1C10 and P1C11 also indicated minor wrap-arounds occurring in the LC × LC system, also illustrating the high sensitivity of the assay. The outstanding performance of the LC × LC system in terms of retention time stability in both dimensions was demonstrated in our previous work,[11] ensuring highly repeatable and accurate fractionation.

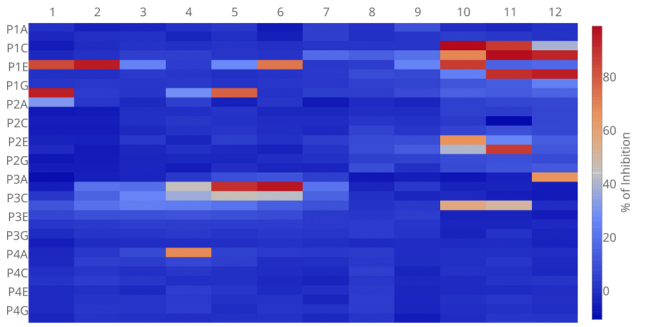


Figure 2. Average (n = 3) percentage of AChE inhibition of the 384 fractions in 4 96-well plates of the EPA 531.1 carbamate mixture (2 μg/mL of each component) after LC × LC fractionation. The numbers on the top are the column numbers (1−12) of the plates and the letter and number combinations to the left of the graph show the plate number (P1−P4) together with the row number (A-H) of the plates.

LC × LC Separation of the LVSPE Extract of the WWTP Effluent. The LC × LC separation was established in accordance with our previous work.[11] Because of good orthogonality and solvent compatibility, C18 and PFP columns were selected for the two chromatographic dimensions. A fast gradient in the second dimension was optimized. The contour plot of the LC × LC-ESI (+)-ToF MS chromatogram is shown in Figure 3. Using this system, the peak capacity was significantly enhanced compared with 1DLC as extensive separation is achieved in the second dimension. In total, 92 visible peaks were matched using the template matching

DOI: 10.1021/acs.analchem.5b04311
Anal. Chem. 2016, 88, 2353−2360

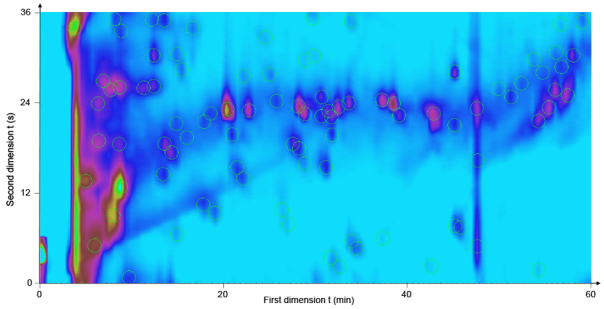


Figure 3. Contour plot of the LC × LC-ESI (+)-ToF MS analysis of the Brno WWTP effluent extract. MS data were acquired at a frequency of 5 Hz. Details of the chromatographic conditions and MS settings are given in the Experimental Section .

function after blob detection (minimum peak 1000, absolute value). The estimated orthogonality was 0.937 (93.7%) using the surface coverage approach[11] and 0.945 (or 94.5%) using the spread of peaks method.[19] The results from the two different methods were comparable and both showed great orthogonality of the LC × LC separation. The enhanced peak capacity and near perfect orthogonality provide the optimal basis for high resolution microfractionation in a relatively short time and supporting fast and accurate identification of active compounds after bioassay screening.

AChE Activity in LC × LC Fractions of Brno WWTP Effluent Extract. For the assessment of AChE activity in the extract of the Brno WWTP effluent, the extract and the corresponding blank were fractionated with LC × LC using the same procedure as for the carbamate mixture. Figure 4

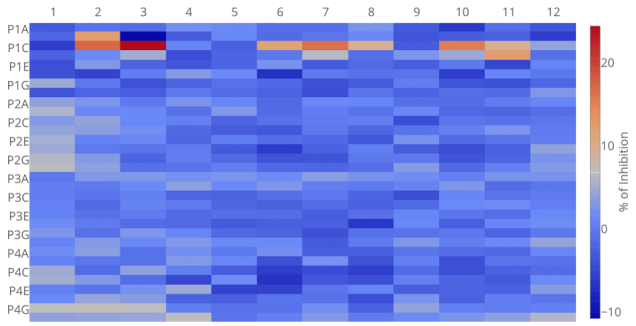


Figure 4. AChE inhibition (%, n = 3) of the 384 fractions of the Brno WWTP effluent extract after LC × LC fractionation. The numbers on the top are the column numbers (1−12) of the plates and the letter and number combinations to the left of the graph show the plate number (P1−P4) together with the row number (A-H) of the plates.

demonstrates the AChE activity in the 384 fractions of the extract after bioassay screening. In total, there were 7 active fractions when a threshold value of 10% inhibition was applied. All active fractions were found in the first well plate containing the more polar compounds, with activities in the range of 11 to 24% inhibition (P1B2, 11.9%; P1C2, 16.9%; P1C3, 24.2%; P1C6, 11.4%; P1C7, 16.1%; P1C10, 15.1%; P1D11, 12.1%). The observation of the concentration of the activity in the more polar fractions of the extract is supported by the fact that the AChE inhibition assay is performed exclusively in the aqueous phase.

Effect-Directed Analysis of the Active Fractions Using Parallel LC × LC-ESI (+)-ToF MS. Using the experimental setup with the postcolumn splitter (Figure 1) it was possible to directly link the active fractions to the chromatographic peaks containing high resolution MS information. The identification of compounds that may have contributed to the activity was therefore rather straightforward. In contrast to the conventional EDA approach in which fractionation into larger fractions is applied, high resolution microfractionation greatly reduced the number of candidate chemicals in the active fractions. Merely 2−4 major MS peaks (intensity >10000) were detected in each active fraction and tentative identification of these peaks was performed according to their accurate masses and isotopic patterns (Table 2). The maximum mass error was set at 10 ppm and the maximum mSigma value (lower indicates a better fit) was 20. For the peaks for which SmartFormula and CompoundCrawler returned more candidates, the log Kow values of the candidate compounds were also taken into account to rank the results. In well P1B2, the major peaks were observed at m/z 125.9875 and m/z 110.0095 but no chemical formula was generated by SmartFormula. In the other six active wells, several pharmaceuticals and their metabolites could be tentatively identified, as shown in Table 2. Among all the tentatively identified compounds, tiapride and lamotrigine were reported to be weak AChE inhibitors with IC_{50} values of 1−10 μM and 50−150 μM, respectively.[22,23] The report of the presence of tiapride in WWTP effluent was rather limited,[24] while lamotrigine was found widely in environmental water samples, including drinking water, surface water and WWTP effluent.[25] Although no AChE inhibiting activity was reported in the literature for amisulpride, the structurally fairly similar antipsychotic drug sulpride, was reported as a weak AChE inhibitor.[22] Therefore, in addition to tiapride and lamotrigine, amisulpride was also selected for further analytical and bioassay confirmation.

Two dimensional retention alignments were performed to confirm the presence of tiapride (m/z 207.1493, $[M + H]^+$), lamotrigine (m/z 242.1438, $[M + H]^+$) and amisulpride (m/z 305.1094, $[M + H]^+$) in the WWTP effluent extract. In Figure 5, LC × LC runs of the three standards are shown at the top, while the EICs of the compounds are shown at the bottom. For all three tentatively identified compounds, the retention times in the first dimension perfectly matched with those of their corresponding standard. In the second dimension, the retention time differences of tiapride, lamotrigine and amisulpride between the standards of and the sample were 1.23 s, 0.00 and 0.21 s, respectively. In earlier work,[11] a maximum retention time deviation of 5% in the second dimension was proposed as a criterion for confirmation, meaning that with a second dimension run time of 36 s the maximum deviation is 1.8 s. Therefore, the presence of the three candidate compounds was confirmed.

Bioactivity Confirmation of Candidate AChE Inhibitors. To confirm the bioactivity in the active fractions, semiquantification based on LC × LC-ToF MS of tiapride, lamotrigine and amisulpride was performed. Semiquantification was performed here as EDA is not particularly suitable for quantitative analysis due to the impossibility to include internal standards. The concentrations in the well of the three identified compounds that exhibited AChE inhibition were determined using calibration curves. In fraction P1C3, the concentration of tiapride was estimated to be 2.3 μM. Due to wrap-around, tiapride could also be found in fraction P1C6 at an estimated concentration of 1.0 μM. In fraction P1C7, where amisulpride and lamotrigine were identified, the estimated concentrations were 1.0 μM and 1.5 μM, respectively. The concentrations of

DOI: 10.1021/acs.analchem.5b04311
Anal. Chem. 2016, 88, 2353−2360

Table 2. Tentative Identification of Compounds Present in the Active Fractions[a]

fraction	*m/z* measured	possible $[M + H]^+$	*m/z* calculated	error (ppm)	mSigma	candidate	logKow (KOWWIN v1.67)	known biological role	known AChE activity
P1C2	232.1080	$C_{12}H_{14}N_3O_2$	232.1081	−0.2	5.9	Isocarboxazid	1.32	nonselective, irreversible monoamine oxidase inhibitor (MAOI)	no
						Triaziquone	0.51	react with DNA to form intrastrand cross-links; used in chemotherapy	no
						4-formylaminoantipyrine	0.50	metabolite of aminophenazone.	no
	246.1238	$C_{13}H_{16}N_3O_2$	246.1237	0.5	15.0	4-acetamidoantipyrine	−0.13	metabolite of metamizole	no
P1C3	309.1810	$C_{16}H_{25}N_2O_4$	309.1809	0.4	8.3	Ubenimex	0.18	competitive protease inhibitor, aminopeptidase B inhibitor, leukotriene A4 hydrolase and aminopeptidase N; used in treatment of myelocytic leukemia	no
	250.1792	$C_{15}H_{24}NO_2$	250.1802	−3.7	12.6	Alprenolol	2.81	nonselective β blocker; used in the treatment of angina pectoris	no
	329.1528	$C_{15}H_{25}N_2O_4S$	329.1530	−0.6	10.5	Tiapride*	0.53	selective D2/D3 dopamine receptor antagonist, used in neurological and psychiatric disorders	yes[22]
P1C6	250.1801 329.1535	wrap-around from fraction P1C3							
	337.2124	$C_{18}H_{29}N_2O_4$	337.2122	0.8	14.0	Acebutolol	1.19	selective β1 receptor blocker for the treatment of hypertension and arrhythmias	no
P1C7	370.1809	$C_{17}H_{28}N_3O_4S$	370.1795	3.8	10.7	Amisulpride*	1.11	an atypical selective D2/D3 dopamine receptor antagonist; used as antipsychotic drugs	no
	268.1908	$C_{15}H_{26}NO_3$	268.1907	0.2	6.1	Metoprolol	1.69	selective β1 receptor blocker used in treatment of high blood pressure	no
	256.0151	$C_9H_8Cl_2N_5$	256.0151	0.0	10.0	Lamotrigine*	0.99	anticonvulsant drug used in the treatment of epilepsy and bipolar disorder	yes[23]
P1C11	268.1910	wrap-around from fraction P1C7							
P1D11	134.0723	$C_7H_8N_3$	134.0713	8.1	5.7	2-aminobenzimidazole	0.88	metabolite of benomyl and carbendazim	no

[a]*Confirmed compounds by analytical standards.

DOI: 10.1021/acs.analchem.5b04311
Anal. Chem. 2016, 88, 2353−2360

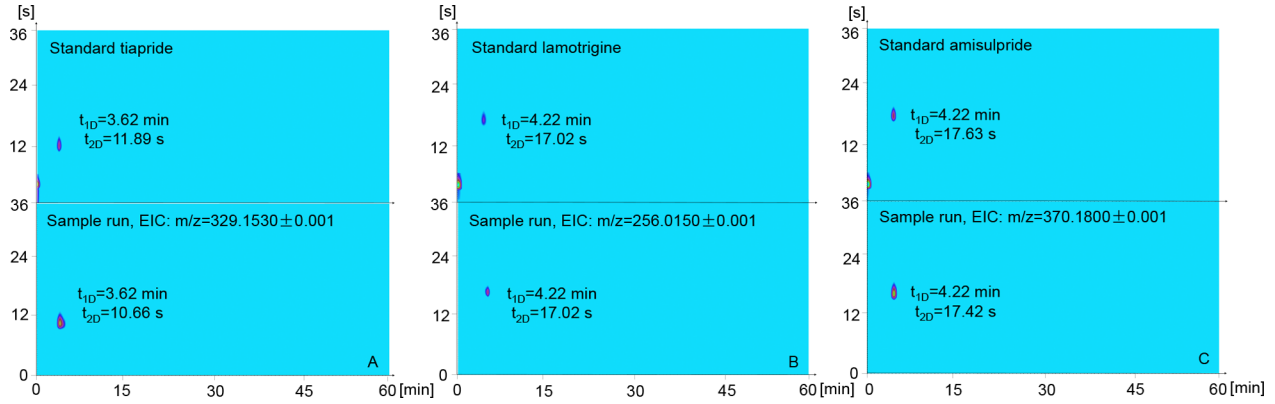


Figure 5. Two dimensional retention alignment of the tentatively identified compounds tiapride (A), lamotrigine (B), and amisulpride (C).

the three compounds at the sampling site (concentration factor =50,000) were therefore approximately 22 ng/l for tiapride, 7 ng/l for amisulpride and 8 ng/l for lamotrigine. For the compounds tiapride and amisulpride, the dose response curves to derive the IC_{50} values in the AChE inhibition assay are presented in Figure 6. Due to the relatively limited solubility of

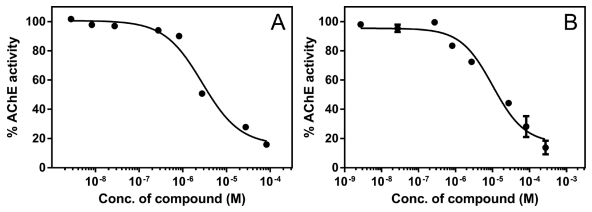


Figure 6. AChE inhibition dose response curves (n = 3) of tiapride (A) and amisulpride (B).

lamotrigine in the test medium, a dose response curve could not be reliably obtained for this compound using the current AChE protocol.

For tiapride, an IC_{50} value of 4.8 μM (3.0−7.6 μM, 95% confidence interval) was found which compares well to the range of 1−10 μM reported in the literature. The IC_{50} of amisulpride was 18 μM (11- 30 μM, 95% confidence interval), which is in the range of the structurally similar compound sulpride (10−100 μM).[22] It is the first time to identify amisulpride as an AChE inhibitor from WWTP effluent.

To evaluate the contribution of the identified compounds to the observed activities in the "hot" fractions, the percentage inhibition could be derived from the dose response curves by interpolation using the estimated concentrations obtained through the semiquantitative chemical analysis. In the fractions P1C3 and P1C6, the respective concentrations of 2.3 and 1.0 μM of tiapride were able to cause 25% and 12% inhibition, which is similar to the measured value 24% and 11%, respectively. Similarly, in fraction P1C7, 1.0 μM of amisulpride showed an inhibition of 12% of the AChE activity, while the measured response is 16%. Care should be taken to interpret this slight discrepancy not too strictly with regard to the percentage of the activity that can be explained by identified compounds, as most EDA studies have a somewhat semi-quantitative aspect. However, considering that in the same fraction the weak AChE inhibitor lamotrigine was present at an estimated concentration of 1.5 μM, this may add to the explanation of the observed effect.

By effect-directed analysis using LC × LC fractionation, a high throughput in vitro AChE inhibition assay and parallel identification by ToF-MS, the identification and confirmation of three pharmaceuticals with applications in neurology/psychiatry was achieved. The compounds causing the effects in the other four active fractions could not be identified, possibly due to the limited sensitivity and/or suitability of the ESI-ToF-MS instrumentation used. For instance, organophosphate pesticides are strong AChE inhibitors, but the preferred interface for their analysis is atmospheric pressure chemical ionization (APCI).[26] Ideally, to achieve a higher identification success rate the suspect screening based on accurate mass, as shown here using ToF-MS instrumentation, should be complemented with MS/MS and in silico fragmentation based approaches for the identification of unknowns.[27]

CONCLUSION

A high throughput EDA method was established for fast screening of AChE inhibitors in WWTP effluents, building further on our previous LC × LC-ToF MS application for environmental analysis.[11] The EDA approach comprised LC × LC separation, ESI-ToF MS detection, and parallel microfractionation followed by high throughput bioassay screening. The analytical performance of the system was evaluated for carbamate pesticides. The bioactivity observed in the 4 × 96 wells correlated excellently with the identity of the active compounds in these fractions.

The presence of tiapride, amisulpride and lamotrigine, all three weak AChE inhibitors that are used as antipsychotic medicines in a WWTP effluent was demonstrated. For amisulpride this was the first time that AChE inhibiting activity was observed.

The greater peak capacity and excellent orthogonality (0.937 and 0.945, respectively, estimated by two methods) using a stationary phase combination of C18 and PFP enabled high resolution postcolumn fractionation. The LC × LC separation also resulted in a significantly reduced matrix effect, which supported a fast and simple identification of AChE inhibitors in the active fractions after bioassay screening. In addition, highly accurate two-dimensional retention alignment in combination with bioactivity provided further confidence in the ToF-MS identification using positive ion mode ESI. To compensate for the loss in sensitivity associated with flow splitting, the use of concentrated extracts is required.

DOI: 10.1021/acs.analchem.5b04311
Anal. Chem. 2016, 88, 2353−2360

Other assays with different toxicological end points in microplate format may easily be implemented in this approach. Other chromatographic column combinations may provide a highly orthogonal fractionation system. For instance, for very polar compounds hydrophilic interaction liquid chromatography (HILIC) may be of interest. Different ionization sources such as APCI and APPI (atmospheric pressure photoionization) which are more efficient at ionizing nonpolar compounds may also be used.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04311.

Additional information as noted in the text (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: xiyu.ouyang@vu.nl; ouyxy1988@gmail.com. Tel.: +31 205989571. Fax: +31 205988941.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Authors are grateful to EU FP7 EDA-EMERGE project (EU contract 290100) for supporting this study.

■ REFERENCES

(1) Brack, W. *Anal. Bioanal. Chem.* **2003**, *377* (3), 397−407.
(2) Legler, J.; van Velzen, M.; Cenijn, P. H.; Houtman, C. J.; Lamoree, M. H.; Wegener, J. W. *Environ. Sci. Technol.* **2011**, *45* (19), 8552−8558.
(3) Thomas, K. V.; Langford, K.; Petersen, K.; Smith, a. J.; Tollefsen, K. E. *Environ. Sci. Technol.* **2009**, *43* (21), 8066−8071.
(4) Yue, S.; Ramsay, B. A.; Brown, R. S.; Wang, J.; Ramsay, J. A. *Environ. Sci. Technol.* **2015**, *49* (1), 570−577.
(5) Bandow, N.; Altenburger, R.; Streck, G.; Brack, W. *Environ. Sci. Technol.* **2009**, *43* (19), 7343−7349.
(6) Li, X.; Stoll, D. R.; Carr, P. W. *Anal. Chem.* **2009**, *81* (2), 845−850.
(7) Nägele, E.; Vollmer, M.; Hörth, P. *J. Chromatogr. A* **2003**, *1009* (1−2), 197−205.
(8) Stoll, D. R.; Talus, E. S.; Harmes, D. C.; Zhang, K. *Anal. Bioanal. Chem.* **2014**, 13−15.
(9) Tranchida, P.; Dugo, P.; Dugo, G.; Mondello, L. *J. Chromatogr. A* **2004**, *1054* (1−2), 3−16.
(10) van der Horst, A.; Schoenmakers, P. J. *J. Chromatogr. A* **2003**, *1000* (1−2), 693−709.
(11) Ouyang, X.; Leonards, P.; Legler, J.; van der Oost, R.; de Boer, J.; Lamoree, M. *J. Chromatogr. A* **2015**, *1380*, 139−145.
(12) Haun, J.; Leonhardt, J.; Portner, C.; Hetzel, T.; Tuerk, J.; Teutenberg, T.; Schmidt, T. C. *Anal. Chem.* **2013**, *85* (21), 10083−10090.
(13) Soreq, H.; Seidman, S. *Nat. Rev. Neurosci.* **2001**, *2* (4), 294−302.
(14) Fukuto, T. R. *Environ. Health Perspect.* **1990**, *87*, 245−254.
(15) Bartus, R.; Dean, R.; Beer, B.; Lippa, A. *Science (Washington, DC, U. S.)* **1982**, *217* (4558), 408−414.
(16) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7* (2), 88−95.
(17) Hamers, T. *Toxicol. Sci.* **2000**, *58* (1), 60−67.
(18) Schulze, T.; Krauss, M.; Novak, J.; Hilscherova, K.; Ait-aissa, S.; Creusot, N.; Macova, M.; Neale, P.; Escher, B. I.; Gomes, T.; Tollefsen, K. E.; Tarcai, Z.; Shao, Y.; Deutschmann, B.; Seiler, T.; Hollert, H.; Tarabek, P.; Tousova, Z.; Slobodnik, J.; Walz, K.; Brack, W. *Jt. Danube Surv. 3* **2014**, 284−295.
(19) Camenzuli, M.; Schoenmakers, P. J. *Anal. Chim. Acta* **2014**, *838*, 93−101.
(20) Booij, P.; Vethaak, A. D.; Leonards, P. E. G.; Sjollema, S. B.; Kool, J.; de Voogt, P.; Lamoree, M. H. *Environ. Sci. Technol.* **2014**, *48* (14), 8003−8011.
(21) Perkins, E. J.; El-Alfy, A.; Schlenk, D. *Toxicol. Sci.* **1999**, *48* (1), 67−73.
(22) Fontaine, J.; Reuse, J. *Eur. J. Pharmacol.* **1980**, *68*, 55−60.
(23) Waldmeier, P. C.; Baumann, P. A.; Wicki, P.; Feldtrauer, J. J.; Stierlin, C.; Schmutz, M. *Neurology* **1995**, *45* (10), 1907−1913.
(24) Wode, F.; van Baar, P.; Dünnbier, U.; Hecht, F.; Taute, T.; Jekel, M.; Reemtsma, T. *Water Res.* **2015**, *69*, 274−283.
(25) Ferrer, I.; Thurman, E. M. *Anal. Chem.* **2010**, *82* (19), 8161−8168.
(26) Thurman, E. M.; Ferrer, I.; Barceló, D. *Anal. Chem.* **2001**, *73* (22), 5441−5449.
(27) Schymanski, E. L.; Singer, H. P.; Longrée, P.; Loos, M.; Ruff, M.; Stravs, M. a.; Ripollés Vidal, C.; Hollender, J. *Environ. Sci. Technol.* **2014**, *48* (3), 1811−1818.

DOI: 10.1021/acs.analchem.5b04311
Anal. Chem. 2016, 88, 2353−2360