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Comprehensive two-dimensional liquid chromatography coupled to high resolution time of flight mass spectrometry for chemical characterization of sewage treatment plant effluents☆

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ABSTRACT

For the first time a comprehensive two-dimensional liquid chromatography (LC × LC) system coupled with a high resolution time-of-flight mass spectrometer (HR-ToF MS) was developed and applied for analysis of emerging toxicants in wastewater effluent. The system was optimized and validated using environmental standard compound mixtures of e.g. carbamate pesticides and polycyclic aromatic hydrocarbons (PAHs), to characterize the chromatographic system, to test the stability of the retention times and orthogonality. Various stationary phases in the second dimension were compared for the LC × LC analysis of silicon rubber passive sampler extracts of a wastewater effluent. A combination of C18 and Pentafluorophenyl (PFP) was found to be most effective. Finally, the hyphenation of LC × LC with HR-ToF MS was optimized, including splitter settings, transfer of data files between the different software packages and background subtraction using instrument software tools, after which tentative identification of 20 environmental contaminants was achieved, including pesticides, pharmaceuticals and food additives. As examples, three pesticides (isoproturon, terbutryn and diazinon) were confirmed by two-dimensional retention alignment.

1. Introduction

Due to massive human activities such as agriculture, wastewater discharges and industrial manufacturing, potentially harmful chemicals reach the environmental. These chemicals are large in number and possess diverse physicochemical properties, ranging from non- or weakly polar compounds such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) to strongly polar compounds such as novel pesticides, pharmaceuticals and personal care products (PPCPs).

Consequently, the analysis of environmental contaminants has always been a challenge and due to the increase in the use and production of chemicals, development of methods for environmental analysis are rapidly expanding. Gas chromatography (GC) in combination with mass spectrometry (MS) has proven its suitability, resulting in the routine separation and identification of environmental contaminants, greatly owing to the well-established mass spectral databases such as the National Institute of Standards and Technology (NIST) database. In the early 1990s, comprehensive two-dimensional gas chromatography (GC × GC) was developed, demonstrating outstanding capability to separate complex environmental samples due to greater peak capacity [1]. Yet, GC and GC × GC are not capable of direct analysis of non-volatile and thermo-labile compounds, unless derivatization steps are introduced, which sometimes are time-consuming.

As an alternative approach to GC, high performance liquid chromatography (HPLC) has also found wide application in the field of environmental chemical analysis over the last decades. Besides the 'classical' reversed phase liquid chromatography (RPLC), in recent years hydrophilic interaction liquid chromatography (HILIC) has been established as a complementary method in environmental analysis. HILIC is especially powerful for separating polar compounds that are usually not retained in RPLC, such as pharmaceuticals [2], organophosphorus pesticides [3], drugs of abuse [4], etc. Besides, by coupling HPLC with modern high resolution mass

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spectrometry (HRMS), non-target screening can be performed for identification and structure elucidation of unknown compounds [5]. Moreover, in the field of effect-directed analysis (EDA) [6], which combines fractionation procedures, effect-based testing and chemical analysis, the use of LC techniques for fractionation of extracts is highly favored over GC due to the complexity of collecting gaseous fractions, although innovation in GC fractionation has been reported [7].

There is no universally applicable separation technology for analyzing all sorts of contaminants, as different classes of compounds require a different approach. However, there is a great demand to apply separation approaches that are able to provide greater separation power, to cover a wider spectrum of analytes and to deliver high throughput.

Comprehensive two-dimensional liquid chromatography (LC × LC) is an emerging technique that has been applied in areas of notable sample complexity, due to its greater peak capacity and tunable selectivity by different stationary phase combinations. Ideally, the peak capacity of LC × LC can achieve the product of each individual peak capacity of the first and second dimension separation, provided that the two dimensions are orthogonal [8]. According to the physicochemical properties of the compounds to be analyzed, the mechanisms of the two separation dimension can be chosen from a variety of stationary phases: RPLC, normal phase liquid chromatography (NPLC), size exclusion chromatography (SEC), ion exchange chromatography (IEC) and HILIC [9]. Examples of LC × LC applications consist of the analysis of synthetic polymers [10], oligonucleotides [11], peptides [12] and proteins [13], pharmaceuticals [14], natural products such as food [15] and Chinese medicines [16] and many other compounds [9].

It is surprising that to our knowledge no LC × LC analysis of contaminants in environmental sample has been reported, although the technique provides almost all the advantages and perfectly meets all the analytical challenges: higher peak capacity, multi-selectivity, suitability for emerging thermo-labile and polar compounds, possibility of post-column fractionation, etc. The objective of this study was to establish and validate an LC × LC-UV/HRMS platform capable of screening and analyzing environmental contaminants in exceedingly complex environmental samples (e.g. wastewater effluent, sediment and biota samples). In our study, the LC × LC system was first validated by environmental standard mixtures. The LC × LC-ToF MS system was then optimized and used for comprehensive analysis of a passive sampler extract of effluent from a wastewater treatment plant (WWTP) in The Netherlands.

2. Experimental

2.1. Sample collection and preparation

The sample used to demonstrate the suitability of LC × LC for environmental contaminant analysis was collected from WWTP Cuijk (The Netherlands) using silicon rubber passive samplers (six blades on holder, 20 g total weight) [17]. The samplers were deployed at the sedimentation pond that receives the effluent from the WWTP. The silicon rubbers were collected after six weeks of exposure and thereafter cleaned with water from the sampling site in order to remove sedimentation and bio-fouling. Cleaned samplers were transported to the lab in plastic containers, and stored at −20 °C until extraction.

When preparing the sample, the silicon rubber blades were cut into small pieces and collected in pre-cleaned thimbles used in the Tecator® Soxtec Avanti extraction system. The extraction was performed with 80 ml of a methanol:acetonitrile (1:2, v/v) mixture and 5–6 boiling stones. The extraction program was 120 min of boiling at 120 °C, 30 min of rinsing, 5 min of recovery and 1 min drying. After cooling the extracts were filtered over Duran® glass fibre filters (100–160 μm) and collected in 250 ml glass bottles. Extraction jars were rinsed twice with 10 ml of extraction mixture. Extracts were evaporated by a TurboVap® at 45 °C to approximately 5 ml. The extracts were transferred (rinsing twice with 5 ml extraction mixture) to conical tubes and evaporated under nitrogen to exactly 10 ml. Before injecting into the LC × LC-ToF MS system, the extracts were diluted by a factor of 10 (v/v) in acetonitrile and Milli-Q water (1:1, v/v).

2.2. Chemicals

Methanol and acetonitrile were of 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 as LC-MS eluent additive was purchased from Sigma-Fluka (Zwijndrecht, The Netherlands).

The EPA 531.1 carbamate mixture was purchased from Sigma-Fluka. The EPA 8270C multiple component standard mixture solution was from Chiron (Trondheim, Norway). The triazine and urea pesticide mixture was obtained from LGC Standards (Teddington, UK). Details of all standard mixtures were given in supplementary information. All standards were diluted to 1 μg/ml prior to injection.

2.3. Instrumentation

The LC × LC system consisted of an Agilent 1100 auto sampler, an Agilent 1100 HPLC binary pump for the first dimension, an Agilent 1290 infinity UHPLC binary pump for the second dimension and an Agilent 1290 infinity thermostatted column compartment (TCC) with a 2-position/4-port duo valve installed as the 2D interface (Agilent Technologies, Waldbronn, Germany). Two sampling loops (60–120 μl) were applied to collect the eluent from the first dimension and thereafter via valve switching, deliver the collected eluent to the second dimension separation in the next sampling cycle. The 2D-LC add-on for Chemstation version B.04.03 (Agilent Technologies) was used to control the LC × LC modulation. The detection was done using an Agilent 1260 Infinity VWD detector (Agilent Technologies) and a Bruker micrOTOF™ Time of Flight (ToF) mass spectrometer with an electrospray interface (ESI, Bruker Daltonics, Bremen, Germany), by splitting the flow after the second dimension using a QuickSplit™ adjustable flow splitter (Richmond, CA, USA). The Bruker micrOTOF™ was initiated (start and stop signal) by external control via a serial port of the auto-sampler and the MS data were recorded by Bruker OtofControl 3.0. Two-dimensional data (both from UV and MS) evaluation was done with the software GC Image 2.3b4 (Lincoln, NE, USA). Compound screening and identification were carried out using the instrument software packages *DataAnalysis* version 4.1 and *MetaboliteDetect* version 2.0 (Bruker Daltonics).

In the first dimension of the LC × LC system, a ZORBAX Eclipse Plus (1.8 μm, 2.1 × 150 mm ID) C18 Rapid Resolution HD column (Agilent Technologies, USA) was used. An Agilent Poroshell 120 (2.7 μm, 50 × 4.6 mm ID) Phenyl Hexyl column (Agilent Technologies, USA), an Agilent ZORBAX (1.8 μm, 50 × 3.0 mm ID) HILIC Plus column (Agilent Technologies, USA) and a Phenomenex Kinetex (2.6 μm, 50 × 4.6 mm ID) PFP column (Phenomenex, USA) were used for the second dimension.

2.4. Methods

2.4.1. LC × LC-ToF MS conditions

The chromatographic conditions of the LC × LC experiments are listed in Table 1. Shifted gradients were applied to provide better

Table 1
LC × LC condition.

Columns	First dimension LC conditions	Second dimension LC conditions
C18 × PFP, 25 °C	Mobile phase: (A) water; (B) acetonitrile. Gradient: 0 min 40% B, 25 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: 1 min. Gradient: 0 min 35% B, shifted to 45% in 25 min and then 70% at 40 min; 0.8 min 45% B, shifted to 65% in 25 min and then 90% at 40 min. Flow rate: 2.0 ml/min.

separation by increasing orthogonality [18]. The splitter was set to divide the post-column flow by 1:4. The lower flow rate was introduced into the MS while the higher flow rate was directed to the UV detector. The resolving power of the mass spectrometer was 10,000. The scan frequency was set to 5 Hz in order to obtain enough data point for fast separation of the second dimension. The ion source and transfer settings of the ESI-ToF MS were optimized to select the mass range and to achieve optimum sensitivity. Measurements were carried out in the positive mode with a scan range from *m*/*z* 50 to 1000. The capillary voltage was 4500 V with end plate offset −500 V. 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. 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 pre-pulse storage was set to 1 μs to enable the detection of smaller molecules.

2.4.2. Data processing and visualization

The LC × LC data was processed by the GC Image software. The total ion chromatograms (TICs) obtained were first calibrated internally by creating a calibration segment prior to the analysis using the calibration tunemix solution on high precision calibration (HPC) in *DataAnalysis*. After calibration, the blank run with identical chromatographic condition was subtracted by *MetaboliteDetect*. Finally, the obtained chromatograms were converted to netCDF files by *DataAnalysis* and imported to GC Image software for visualization. For orthogonality calculation, data were processed in *OriginPro 9* (Originlab, MA, USA) and *Excel* (Microsoft, WA, USA).

3. Results and discussion

3.1. LC × LC system characterization

The reproducibility of the LC × LC system was validated by EPA 531.1 carbamate mixture, as retention time alignment is a crucial part of the identification of unknown contaminants in environmental samples. The retention stability was found to be very stable for both dimensions. Also, to demonstrate the applicability of LC × LC in environmental analysis, estimate the orthogonality of LC × LC separation after injecting an EPA 8270c multiple component standard mixture, a surface coverage metric was applied. Detailed description for test of retention stability and orthogonality were provided in supplementary information.

3.2. LC × LC analysis of environmental samples

3.2.1. Stationary phase selection

As environmental contaminants are often small and neutral molecules, SEC and IEC were excluded from the selection of the two dimensions. In addition, as the mobile phases in the two dimensions are intrinsically incompatible, the coupling of NPLC to RPLC needs a sophisticated engineered interface such as a solvent evaporation device [1], which may cause sample loss for volatile and themoliable compounds. Therefore, our research was focused on the combinations RPLC × RPLC and RPLC × HILIC. Because of relatively universal selectivity and high plate counts, a C18 column was employed as the first dimension. For the second dimension either HILIC or RPLC with a different stationary phase were selected. A Phenyl-hexyl column has a different separation mechanism of π-π interaction for PAHs and some other aromatic compounds, which are of high environmental concern. A Pentafluorophenyl (PFP) column provides strong steric interaction as well as a unique selectivity for halogenated compounds [19], such as organochlorine pesticides (OCPs), some organophosphorus pesticides (OPPs), PCBs and PBDEs. These are notorious environmental contaminants that have been reported as neurotoxicants [20], carcinogens [21], or thyroid hormone disrupting compounds [22]. Recently, PPCPs have drawn increasing attention [23], unlike classical environmental contaminants, most of the PPCPs have significantly polar features, making them very suitable for separation using a HILIC column.

It is essential to perform a fast gradient elution in the second dimension of the LC × LC system, in order to achieve better resolution and to avoid the occurrence of wrap-around [24]. The wrap-around occurs in LC × LC separations when the modulation time is shorter than the compounds' second dimension retention time. The compounds therefore elute to the detector during the next modulation period and may coelute with the compounds in that fraction, which results in a loss of resolution. Modern developments in HPLC column technology offer many possibilities for superfast separation, such as monolithic, core-shell and sub-2-μm particle columns (UPLC/UHPLC). A comparison of the three technologies being applied in ultrafast HPLC indicated that core-shell and sub-2-μm particle columns have better performances than monolithic columns [25,26]. In addition, compared to sub-2-μm particle columns, core-shell particle columns are more tolerable of dirty samples, which is highly important in environmental analysis.

It can be observed from Fig. 1 that the contour plots of the LC × LC chromatograms of the C18 × PFP and C18 × Phenyl-Hexyl column combinations are similar, as both stationary phases have selectivity according to hydrophobicity, though the C18 × PFP combination provides higher peak capacity since more peaks were found to be separated by the second dimension. For C18 × HILIC, an entirely different picture was obtained, as the separation mechanism of HILIC is considered to be multimodal partitioning. Orthogonality was estimated in each case by the surface coverage method (method see in supplementary information). The vectors used to define the effective area of separation in the three experiments were also shown in Fig. 1. The calculated surface coverage was 0.643 for (A), 0.671 for (B) and 0.687 for (C). Solely analysing these values, it seems that the C18 × HILIC combination has the highest orthogonality, which makes sense because of the entirely different separation mechanism of the different phases. However, the LC × LC chromatogram of the C18 × HILIC combination indicates that the number of peaks separated in the second dimension is rather limited compared with the C18 × PFP combination. This can be explained by the nature of the sampling method, as a silicon rubber passive sampler is suitable for the sampling of medium polar and non-polar compounds [27,28]. Therefore, for further LC × LC-HRMS characterization of this wastewater extract, the C18 × PFP combination was finally selected.

3.2.2. LC × LC-ToF MS identification

For an in-depth screening of environmental samples, the coupling of LC × LC separation with HRMS enables the tentative identification of unknown compounds by the determination of their accurate masses. Practically, there are two consequences of the application of MS detection related to the ultra-fast separation

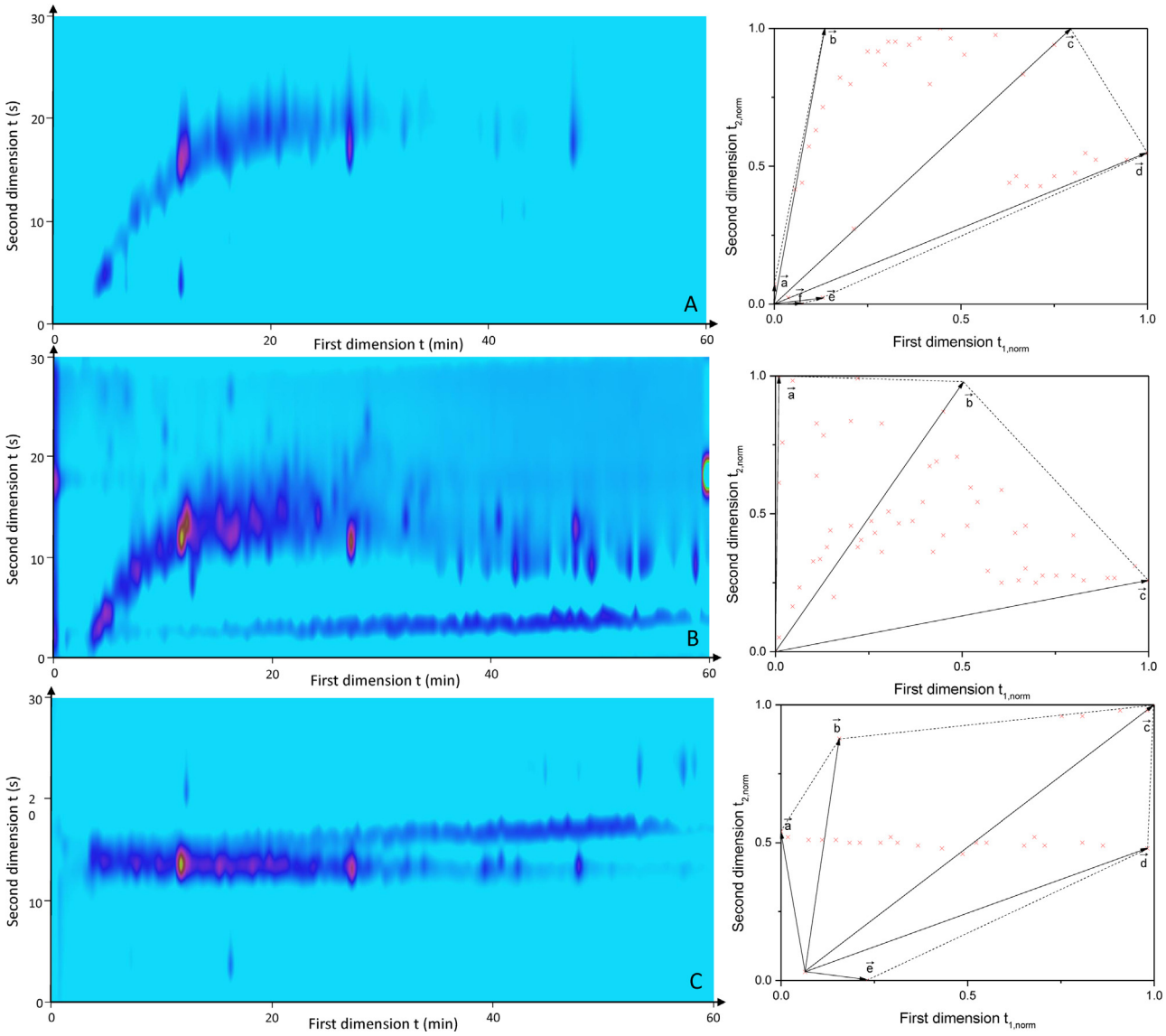

Fig. 1. Contour plot of the LC × LC separation of the Cuijk wastewater extract by different stationary phase combinations: C18 × Phenyl-Hexyl (A), C18 × PFP (B) and C18 × HILIC (C) and their corresponding surface coverage calculation by vectors. Detection was performed using UV at 290 nm with sampling frequency 5 Hz.

in the second dimension of the LC × LC system [29], and that may explain why the number of LC × LC-MS applications is limited compared to LC × LC-UV or LC × LC–diode array detection (DAD). First, a reasonably fast scanning speed, or in other words, high sampling frequency (≥5 Hz) of the MS is required, as enough data points need to be acquired for each peak in order to maintain the resolution that is obtained in the fast separation. Due to its capability of fast scanning and high resolving power, ToF MS is optimal for coupling with LC × LC. Secondly, very high flow rates (≥1 ml/min) are not favored by most of the atmospheric ionization (API) sources available, especially for electrospray (ESI), which covers the broadest range of analytes and is thus the most widely used interface for LC-MS. To avoid significant sensitivity loss due to a high flow rate and minimize possible peak broadening caused by the dead volume, a commercially available post-column splitter was implemented in our system, directing 20% of the flow to the ToF MS.

When comparing with UV detection, the ESI-ToF MS has a considerably higher background when analysing complex environmental samples, and in LC × LC this phenomenon is overemphasized. After blank run subtraction, the visualization was still not optimal. To minimize this effect, several data treatment steps were included in the data analysis workflow. First, the total ion chromatogram (TIC) was dissected to find all the chromatographic peak components with a signal to noise >3 using the instrument's *DataAnalysis* software and roughly 300 compound spectra (peaks in TIC) were generated. For further evaluation, all dissected peaks with an intensity >4000 counts were listed and their *m/z* values were imported into the GC Image software to extract their extraction ion chromatograms (EICs) with an extraction mass window of 1 mDa from the TIC. Finally, a summation of EICs of all peaks with an intensity >4000 was visualized as represented in Fig. 2. In this contour plot 32 peaks were assigned and numbered 1–32.

The 32 peaks in Fig. 2 are spatially distributed in the separation space, indicating that a satisfactory orthogonality was achieved by LC × LC. It is conceivable that peaks such as 5 and 6, 10 and 11, 13–16, 22–24, 25 and 26, 28 and 29 would not be easily separated by conventional LC in a similar time frame. The mass spectra of these peaks are shown in Fig. S4-S7 in the supplementary data. Most of the spectra show only one intense peak, which proves that the ultra-high separation power achieved by LC × LC is preserved after coupling to ToF MS. In addition, comparing with conventional

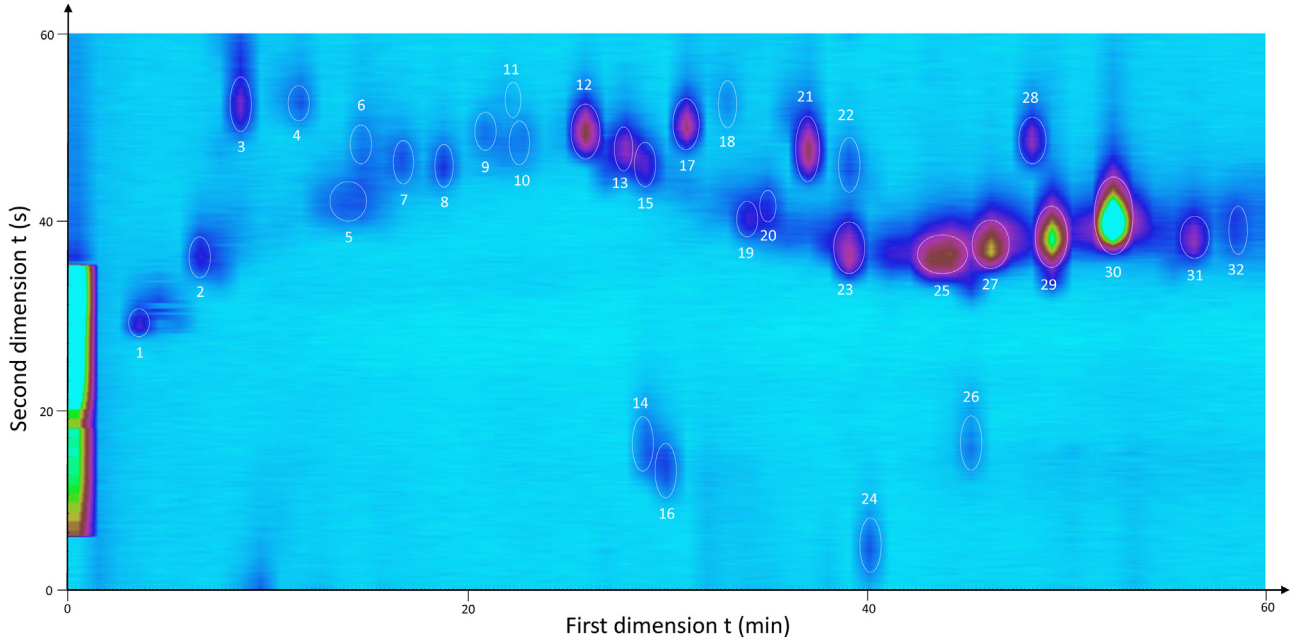

Fig. 2. Contour plot (summation of EICs with intensity >4000 counts) of LC × LC-ESI (+)-ToF MS analysis of the Cuijk wastewater effluent exact. A phase shift of 10s was applied for the second dimension to put all the peaks in the frame. MS data were acquired at a frequency of 5 Hz. Details of the chromatographic conditions and MS settings are given in Section 2.4.1. Peak numbers are explained in Table 2 and the text.

Table 2
Candidate list after tentative identification of compounds present in a wastewater effluent extract after LC × LC-ESI (+)-ToF MS analysis using SmartFormula and CompoundCrawler tools and web based database search.

No.	*m/z* measured	Intensity	Suggested molecular formula $[M+H]^+$ or $[M+Na]^+$	*m/z* calculated	Error (ppm)	mSigma	Tentative candidates	Application/Source
3	188.1434	24745	$C_{13}H_{18}N$	188.1434	0.28	3.5	Selegiline	Drug (Parkinson's disease)
4	202.1225	9828	$C_{13}H_{16}NO$	202.1226	−0.51	7.3	(E)-1-Cinnamoylpyrrolidine	Food and beverage additive
5	156.0815	6944	$C_{11}H_{10}N$	156.0809	3.80	−4.5	4-Phenylpyridine	Beverage additive
6	278.1900	6779	$C_{20}H_{24}N$	278.1903	−1.30	10.1	Amitriptyline or EDDP	Antidepressant/drug Metabolite
7	207.1494	9287	$C_{12}H_{19}N_2O$	207.1492	0.54	8.3	Isoproturon	Herbicide
12	212.2010	8166	$C_{13}H_{26}NO$	212.2009	0.08	9.1	Ethyl menthane carboxamide	Food and beverage additive
14	293.1055	10882	$C_{13}H_{19}ClN_2NaO_2$	293.1027	9.40	12.6	Chloroprocaine	Local anesthetic
15	182.0095	7237	$C_8H_8NS_2$	182.0093	1.35	14.1	2-Methylthiobenzothiazole	Degradation product of biocides
17	232.1334	33911	$C_{14}H_{18}NO_2$	232.1332	0.92	0.7	Indeloxazine	Drug for cerebrovascular disease
18	242.1438	4947	$C_{10}H_{20}N_5S$	242.1434	1.67	6.7	Terbutryn or Prometryn	Herbicide
19	220.1702	6002	$C_{14}H_{22}NO$	220.1696	2.67	17.4	Sedamine or Fabianine	Natural products
	249.1872	7373	$C_{16}H_{25}O_2$	249.1849	9.21	13.5	4,7,10,13-hexadecatetraenoic acid	Fatty acid
20	225.0914	8743	$C_{15}H_{13}O_2$	225.0910	1.57	3.3	Flavanone	Natural product
22	219.1945	16676	$C_{12}H_{27}O_3$	219.1955	−4.55	8.3	8,8-Dimethoxy-2,6-dimethyl-2-octanol	Food additive
	241.1773	10252	$C_{12}H_{26}NaO_3$	241.1774	−0.70	3.2	Na adduct	/
23	226.1336	11064	$C_{14}H_{16}N_3$	226.1339	−1.20	11.6	Cyprodinil	Pesticide
24	443.2555	12354	$C_{28}H_{36}NaO_3$	443.2557	−0.46	10.8	Tingenone	Natural product
26	312.2317	7057	$C_{21}H_{30}NO$	312.2322	−1.66	9.5	Biperiden	Drug (Parkinson's disease)
27	305.1090	34662	$C_{12}H_{22}N_2O_3PS$	305.1088	0.66	5.9	Diazinon	Pesticide
28	322.0665	29395	$C_{16}H_{17}ClNO_2S$	322.0663	0.64	2.8	Clopidogrel	Drug for cerebrovascular disease
29	399.2510	159335	$C_{18}H_{40}O_7P$	399.2506	0.96	4.0	Tris(2-butoxyethyl) phosphate	Organic flame retardant
	421.2326	24812	$C_{18}H_{39}NaO_7P$	421.2326	0.02	2.4	Na adduct	/
30	273.1848	130818	$C_{18}H_{25}O_2$	273.1849	−0.48	6.1	Estradiol	Drug metabolite and food

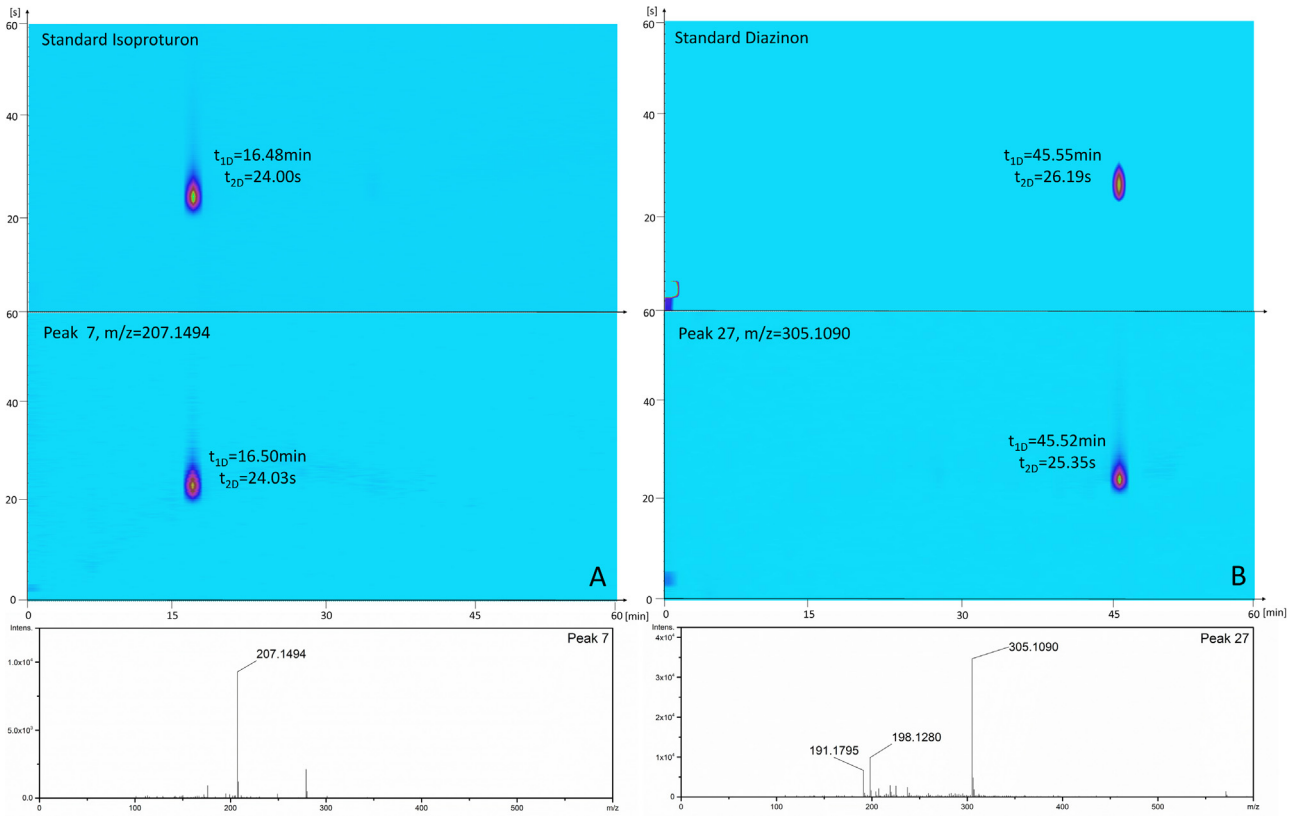

Fig. 3. Retention time alignment of peak 7 (A) and peak 27 (B).

1DLC, the analytes which finally reach the MS interface were further separated by the second dimension and therefore suffered less from ion suppression, which is quite advantageous for samples from complex environmental matrices.

To perform a tentative identification of the 32 peaks, the possible molecular formulas were evaluated by the *SmartFormula* function in *DataAnalysis* based on the accurate mass and isotopic pattern (a lower mSigma value indicating a better fit). In addition to C, H, O, and N, the elements F, Cl, Br, S, P and Si were also taken into account. The nitrogen rule, ring structures and double bonds and the number of carbon atoms were automatically checked by the program. The maximum mass deviation was set at 10 ppm and the threshold of the mSigma value was set at 20. The candidate list of molecular formulas was searched in several databases including ChemSpider, NIST, and METLIN using the *CompoundCrawler* tool in *DataAnalysis* [28]. Of the 32 peaks, 20 highest scored chemical formulas evaluated by the *SmartFormula* function are tentatively identified. The results are shown in Table 2 where possible environmental contaminant compounds assigned to these formulas were given.

To demonstrate the suitability of the described approach for screening and identifying compounds present in WWTP effluents, confirmation experiments were performed for tentatively identified peak 7 ($m/z = 207.1493$, $[M+H]^+$), peak 18 ($m/z = 242.1438$, $[M+H]^+$) and peak 27 ($m/z = 305.1094$, $[M+H]^+$), which were tentatively identified as the biocides isoproturon, terbutryn (or prometryn) and diazinon, respectively. Identical LC × LC chromatographic conditions and the same MS settings were applied for a triazine and urea pesticide standard mixture (29 compounds, containing isoproturon) and a diazinon standard solution. For peak 7 (Fig. 3A), an EIC of $m/z = 207.1490 \pm 0.001$ was generated and compared with the EIC of isoproturon in the standard mixture in GC Image software. Their measured retention times matched perfectly. Also, for peak 27 an EIC of $m/z = 305.1090 \pm 0.001$ was generated and compared with the TIC run of a diazinon standard solution (Fig. 3B). The measured retention time alignment was also excellent.

With respect to peak 18, retention alignment experiments were performed using the same approach. As terbutryn and prometryn have identitical molecular formulas and very similar structures, they have very close retention times. Standard runs of terbutryn and prometryn were carried out separately. The retention times measured were 30.02 min (first dimension), 39.16 s (second dimension) for terbutryn and 28.02 min (first dimension), 41.41 s (second dimension) for prometryn. An EIC of $m/z = 242.1440 \pm 0.001$ was generated from the sample run and the retention times were measured as 30.02 min (first dimension) and 39.57 s (second dimension). Due to the retention time stability in both dimensions of the LC × LC system it may be concluded that peak 18 can only be terbutryn. In conclusion, with the accurate masses (error < 5 ppm), isotopic patterns (mSigma < 10) and retention times ($error_{1D} < 0.03$ min, $error_{2D} < 1.00$ s), isoproturon, terbutryn and diazinon are confidently confirmed in the WWTP effluent by LC × LC-ToF MS.

4. Conclusion

The performance of an LC × LC system was explored with regard to its potential for the analysis of standard mixtures of environmental contaminants and wastewater effluent extracts. Good retention time stability and orthogonality of the system were achieved. In combination with a C18 stationary phase in the first dimension, Phenyl-Hexyl, PFP and HILIC stationary phases were tested as the second dimension for the separation of an extract of an effluent sample by silica rubber passive sampling. The C18 × PFP column combination proved to the most effective combination

for this sample. Although the combination of C18 × HILIC was not suitable for the current sample, it would be still interesting for the samples contain more polar compounds.

To assess the suitability of two-dimensional LC for environmental analysis, LC × LC-ESI (+)-ToF MS was developed to perform non-target screening of sewage treatment plant effluent. LC × LC separation of the sample was found to be highly effective as many separations occurred in the second dimension, which finally led to simpler tentative MS identification. Overall, 20 compounds were tentatively identified in an extract of a wastewater effluent, based on their accurate mass, using easily accessible databases such as *ChemSpider*. To demonstrate the applicability of our identification strategy, for several candidate compounds (i.e., isoproturon, terbutryn and diazinon) analytical confirmation was carried out by comparing their two dimensional retention times in the sample with those observed after injection of the corresponding standards. The presence of isoproturon and diazinon was easily confirmed and the confirmation of terbutryn showed the strength LC × LC being able to discriminate between structurally very similar compounds having identical molecular formulas. The strategy can be applied for confirmation of other tentatively identified compounds in the effluent sample as well as other environmental samples.

Despite the intrinsic suitability of ToF MS detection for coupling to LC × LC through the data acquisition speed, only a few examples of LC × LC-ToF MS applications are reported in the literature. Presumably, this is caused by the lack of appropriate data treatment and visualization software tools for the coupling of two-dimensional LC and HRMS techniques. For visualization purpose, the background extraction, which groups the peaks of a certain range of intensity (>4000 counts) in one chromatogram, was essential to construct a LC × LC-MS plot using the GC Image software for complex environmental samples, indicating the need for improvement of the current LC × LC-MS data analysis software.

In the near future the use of other API sources will be investigated for the established LC × LC-ToF MS system in order to address compounds that are not easily ionized by ESI. In addition, other stationary phase combination of LC × LC will be studied to broaden the application field into analyzing more polar pollutants. Moreover, the current LC × LC system may be fine-tuned for microfractionation into ≥96 well plates, enabling the implementation in EDA studies for comprehensive characterization of toxicity and identity of environmental contaminants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.12.075.

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