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Online and Splitless NanoLC × CapillaryLC with Quadrupole/Time-of-Flight Mass Spectrometric Detection for Comprehensive Screening Analysis of Complex Samples

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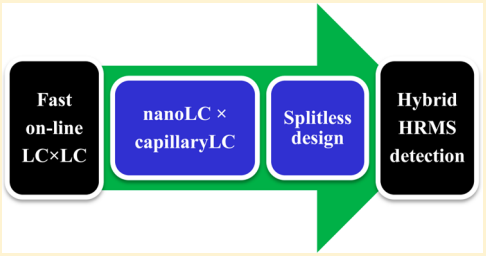
Supporting Information

ABSTRACT: A novel multidimensional separation system based on online comprehensive two-dimensional liquid chromatography and hybrid high-resolution mass spectrometry has been developed for the qualitative screening analysis and characterization of complex samples. The core of the system is a consistently miniaturized two-dimensional liquid chromatography that makes the rapid second dimension compatible with mass spectrometry without the need for any flow split. Elevated temperature, ultrahigh pressure, and a superficially porous sub-3-μm stationary phase provide a fast second dimension separation and a sufficient sampling frequency without a first dimension flow stop. A highly loadable porous graphitic carbon stationary phase is employed in the first dimension to implement large volume injections that help countervailing dilution caused by the sampling process between the two dimensions. Exemplarily, separations of a 99-component standard mixture and a complex wastewater sample were used to demonstrate the performance of the dual-gradient system. In the second dimension, 30 s gradients at a cycle time of 1 min were employed. One multidimensional separation took 80−90 min (~120 min including extended hold and re-equilibration in the first dimension). This approach represents a cost-efficient alternative to online LC × LC strategies working with conventionally sized columns in the rapid second dimension, as solvent consumption is drastically decreased and analytes still are detectable at environmentally relevant concentrations.



Hyphenation of liquid chromatography and mass spectrometry (LC-MS) is widely accepted and used for the analysis of complex environmental samples that can contain hundreds of semi- and nonvolatile substances.[1] For a target screening of a limited number of analytes with reference standards, a sufficient identification of the targets can often be achieved by fragmentation experiments carried out on conventional unit-resolution tandem mass spectrometers (MS/MS). In contrast, the application of hybrid full-scan high-resolution mass spectrometers (hybrid HRMS), such as quadrupole/time-of-flight (QqTOF) or linear ion trap/orbitrap, can help to avoid false positives resulting from the limitations of conventional MS/MS.[2] The additional high mass accuracy and mass resolving power enhance selectivity against the background signals of the matrix and allow the determination of the "exact mass" as well as retrospective data analysis.[2] These benefits of hybrid HRMS are pronounced in the case of screening for suspected or unknown compounds. However, coeluting isobars can still lead to false positive results. Moreover, matrix effects resulting from coelution of a large amount of ionizable matrix compounds can suppress ionization of the analytes in the ion source.[3,4] One approach to minimize coelutions is to simply increase the peak capacity of the chromatographic system, for example, by using ultrahigh-performance liquid chromatography (UHPLC).[5] However, the introduction of additional selectivity in terms of a second chromatographic dimension which helps to separate the analytes from the interfering matrix is a more promising approach for avoiding matrix effects in particular.

In the past decade, comprehensive two-dimensional liquid chromatography (LC × LC) has gained much interest, as can be seen from several reviews.[6−10] This boost in publications on LC × LC surely coincides with the commercialization of promising hardware such as UHPLC systems or the sub-3-μm superficially porous particles.[9] Hyphenation of LC × LC to mass spectrometry (MS) was recently reviewed in a devoted book edited by Mondello.[11,12] In principle, all possible effects and problems known from the hyphenation of one-dimensional liquid chromatography (1D-LC) to mass spectrometry can as well arise for the LC × LC-MS combination, as the MS is still connected to only one liquid chromatographic dimension, which is the second dimension (D2). Depending on the LC ×

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LC mode in use, however, the operating conditions of the D2 can be dictated by the Murphy−Schure−Foley (M−S−F) criterion that every peak of the first dimension (D1) has to be sampled about three to four times for a low-loss conservation of the D1 separation.[10,13] This rule does not necessarily affect D2 conditions of offline-operated LC × LC systems, where there is no time dependence between both LC dimensions.[14−17] Nevertheless, it means a major constraint to systems used in the online mode, especially in the case of a fast single-column D2 system. Here, the maximum D2 run time is defined by the time in which one fraction of the D1 effluent is collected.[10] Thus, to fulfill the M−S−F criterion, D2 speed has to be very fast, otherwise resolution is decreased by undersampling.[9] Therefore, much attention is given to shorten D2 run times that are typically around 20 to 120 s. Speed is usually gained by the application of flow rates as high as 1 to 5 mL min^{-1} on short D2 columns with diameters of 1 mm and above.[10−12] Apart from the economically inefficient solvent consumption, a flow splitter is necessary to avoid overloading the ion source of the mass spectrometer. Upper tolerable flow rate limits of common electrospray ionization (ESI) sources are approximately 1 mL min^{-1}, and for atmospheric pressure chemical ionization (APCI), they are 2 mL min^{-1};[11] however, these values are already far above the optimum. The necessity for a flow split inevitably adds losses of sensitivity.[18]

Besides the possible interface overload, the practitioner has to pay attention to the data acquisition rate of the respective mass spectrometer, especially if older quadrupole-based MS systems are used, as resulting peak widths for short D2 runs can be 1−2 s and below.[19] Although fast-scanning systems of the latest generation may be capable of measuring the minimum-needed 6 to 10 data points per peak under certain conditions (e.g., reduced mass-to-charge ratio (m/z) range), it is recommended in the literature to switch to time-of-flight (TOF) type MS as the high data acquisition rates are more than sufficient and independent of the selected m/z range.[11,18] The application of multiple D2 systems operated in parallel allows decreasing the critical D2 speed;[20−22] however, besides increased complexity, additional hardware such as columns, valves, and costly MS detectors would be necessary. The alternate use of stop-flow methods also allows for a sufficient number of D1 eluate samples, but overall analysis time is drastically increased because the D1 has to be stopped after each eluate sample to allow the D2 separation to be performed.[23−26]

A different way to decrease the solvent load to the mass spectrometer is the miniaturization of the D2 to capillary LC. Miniaturized LC × LC systems with MS detection mainly originate from the field of proteomics.[27−32] In order to achieve high peak capacities, most of them are either operated in (automated) offline mode,[30] in stop-flow mode[29] or use a step-gradient[27,31,32] ion exchange chromatography (IEC) in D1 to allow independently long D2 run times. To the best of our knowledge, the only hitherto reported online LC × LC system with miniaturized D2 was developed by Opiteck et al., who applied IEC in D1 and reversed-phase chromatography in D2 to separate protein mixtures within two hours (48 fractions, 150 s each).[28]

The goal of the present work was to develop a multidimensional system for the qualitative screening analysis of complex samples on the basis of a consistently miniaturized, online dual-gradient LC × LC system hyphenated to hybrid HRMS detection. The system should feature a large volume injection and a completely splitless design to increase sensitivity and prevent sample losses. Supporting technologies known from nonminiaturized online LC × LC were used to increase second dimension speed, among those elevated temperature,[10,33,34] ultrahigh pressures[35,36] and a superficially porous sub-3-μm stationary phase.[36,37]

MATERIALS AND METHODS

HPLC Instrumentation. As a development platform for the LC × LC part of the multidimensional system, an Eksigent NanoLC-Ultra 2D pump system (AB Sciex, Dublin, CA) was used. Its operation was controlled by a PC and Eksigent software version 3.12.1. Apart from a column oven compartment with two integrated 10-port, 2-position valves, the Eksigent system contains two binary-gradient pneumatic pumps which can be variably adjusted to different nano- and microflow ranges by exchanging the respective flow module. The standard nanoflow module (50−500 nL min^{-1}) was applied for the D1 gradient pump. In the case of the D2 pump, the standard flow module was replaced by a module with a flow range of 5−50 μL min^{-1}. As flow delivery is performed without splitting, gradients can reliably be mixed at back pressures of up to ∼690 bar (10 kpsi).

A superficially porous sub-3-μm stationary phase was used for the packing of the D2 column. Therefore, 2.6 μm SunShell C18 particles (ChromaNik Technologies, Osaka, Japan) were packed into a custom 50 mm × 0.3 mm inner diameter (i.d.) column by Grace Davison (Worms, Germany). A Hypercarb column (50 mm × 0.1 mm i.d., 5 μm particles, Thermo Fisher Scientific, Dreieich, Germany) was used in D1.

The air-bath oven was used to apply an isothermal temperature of 60 °C with an accuracy of ±1 °C on both columns and all other parts inside the compartment. The two integrated 10-port, 2-position valves were not designed to be constantly operated at elevated temperature. In order to avoid severely increased rotor abrasion, two custom replacement valves (C72NX-4670DY-EKS, Cheminert Nanovolume, 100 μm port bore size, 1/32″ fittings, VICI International, Schenkon, Switzerland) were mounted to the actuators.

MS Instrumentation. The hybrid HRMS instrument used for the implementation of the multidimensional system was a TripleTOF 5600 by AB Sciex (Darmstadt, Germany) equipped with a DuoSpray ion source and a TurboIonSpray probe for ESI experiments. For the sake of low dead volumes between D2 column and detection, the mass spectrometer was modified. The standard emitter tip inside the TurboIonSpray probe was replaced by an emitter with an i.d. of 50 μm. As recommended by the manufacturer, grounding was achieved by clipping a grounding cable on one end to the slots of the source housing and on the other end directly to the inlet steel union of the ion source. MS data acquisition was controlled using a separate PC with AB Sciex Analyst TF 1.5.1. Acquired data including mass spectra were analyzed using the AB Sciex PeakView 1.2.0.1 software out of the D2 raw data.

Capillary System, Modulation, and Injection. The design of the capillary system of the multidimensional system is schematically illustrated in Figure 1.

As is often found in the literature,[18,33,38−42] the online LC × LC interface consists of a two-loop modulation composed of a single two-position 10-port valve. In contrast to many interfaces of this type, counter-flow emptying of one of the two loops is used for the benefit of equal capillary volumes in D2 comparing both valve positions. Regarding the increased influence of extra-

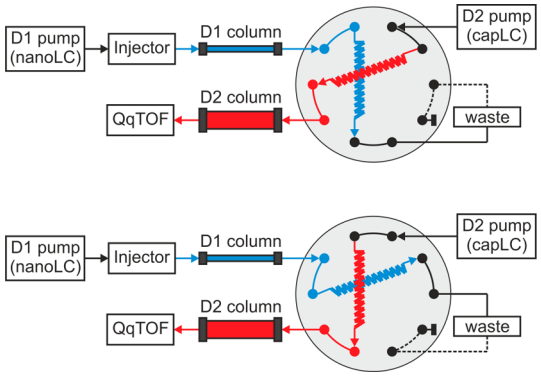

Figure 1. Scheme of the capillary system for both modulation valve positions. Blue: sample path in first dimension. Red: sample path in second dimension.

column volumes in miniaturized LC, this modification prevents retention time shifts between two consecutive D2 runs caused by an otherwise necessary port-to-port bridging capillary. In order to implement the modulation scheme of Figure 1, the left 10-port valve of the Eksigent system was used as an injector to D1, and the right valve was used as the modulation valve. The injection loop to D1 was a 20 cm, 100 μm i.d. PEEKsil capillary (Upchurch Scientific, Oak Harbor, WA) with a calculated volume of 1.57 μL. Prior to each 2D analysis, this sampling loop was manually filled by using a Hamilton syringe (Bonaduz, Switzerland) that was mounted to the injection valve via a capillary and a Luer lock adapter. All connection capillaries of the capillary system including both modulation loops were 360 μm outer diameter (o.d.) fused-silica purchased from Postnova Analytics (Landsberg, Germany), and they were cut to size by a Shortix GC (SGT, Middelburg, The Netherlands) rotating diamond cutter. Inner diameters of the connection capillaries were 25 and 50 μm for the first and second dimension, respectively. Both modulation loops had an equal length of 15.5 cm and an i.d. of 50 μm to avoid additional pressure drop due to restriction of the D2 pathway. The compact arrangement of pump outlets and switching valves inside the air-bath column oven compartment resulted in low extra-column dead volumes throughout the whole capillary system. Accordingly, low gradient delay volumes—calculated from the gradient mixing tee to the head of the column—were established, ~85 nL and ~1 μL for D1 and D2, respectively. In the case of D1, the injection loop does not contribute to gradient delay, as it is switched out of flow prior to gradient start.

Solvents. Ultrapure water (J.T. Baker, LC/MS reagent grade) was purchased from Mallinckrodt Baker (Griesheim, Germany). Acetonitrile and methanol were both LC-MS Optigrade from Promochem (LGC Standards, Wesel, Germany). All LC eluents were acidified by adding 0.1% formic acid (FA) by volume (puriss. p.a., ~ 98%, Sigma-Aldrich, Schnelldorf, Germany).

Standard Mix and Real Sample. Ninety-nine substances were chosen as targets for a suspected screening of wastewater samples. These included 73 pharmaceutical drugs (among others: antibiotics, cytostatics, X-ray contrast media, steroids, beta blockers, and psychotropics), 4 metabolites of sulfonamide antibiotics, 13 pesticides, 6 mycotoxins, and 3 corrosion inhibitors. A detailed list of all substances is provided in Supporting Information Table S-1. Several of them are toxic, cytotoxic, carcinogenic, or possess other harmful properties. The safety data sheets provide details on handling and discharge. A multicomponent test mix containing each target in a mass concentration of 1 μg mL^{-1} was prepared in water/acetonitrile (95:5, v/v) acidified by 0.1% FA.

Groskreutz et al. recently confirmed the applicability of a new 2D-LC technique by using wastewater as a model matrix.[43] Analogously, the complex sample that was exemplarily analyzed to demonstrate the performance of our approach was a 200 mL wastewater sample taken at the inlet of the secondary (or biological) treatment step of a municipal sewage plant. Since this sample type contains very harmful substances as well as pathogenic bacteria and viruses, corresponding laboratory equipment and safety measures are needed. Sample pretreatment prior to the 2D analysis included an adjustment of the pH to 3 using hydrochloric acid, the addition of deuterated internal standards, and solid-phase extraction (SPE) through a 200 mg Oasis HLB cartridge (Waters, Eschborn, Germany). The internal standards, 100 ng each, were bisoprolol-*d*5 (LGC Standards), isoproturon-*d*6 (Pestanal, Sigma-Aldrich), and racemic metoprolol-*d*7 (Campro Scientific, Berlin, Germany). The SPE contained the following steps: conditioning, 5 mL methanol (5 min residence time); equilibration, 5 mL of water at pH 3; sample application; washing, 5 mL of a water/methanol mixture (95:5, v/v); drying for 5 min; elution, 3 × 3 mL of methanol; the eluent was evaporated in a nitrogen stream at 40 °C, and the residue was redissolved in 2 mL of water/acetonitrile (95:5, v/v) acidified by 0.1% FA.

Both the test mix and the pretreated wastewater sample were filtered through a 0.2 μm CHROMAFIL RC 20/25 disposable syringe filter by Macherey-Nagel (Düren, Germany) prior to injection. Between the injections of wastewater samples, it is recommended to measure at least one blank as strongly retained substances may disturb the analysis of the next sample.

First Dimension Gradient Programming and Injection. In the first dimension, a water + 0.1% FA (eluent A)—methanol + 0.1% FA (eluent B) gradient at a flow rate of 200 nL min^{-1} was applied according to the following program: 8 min hold at 1% B, 1—99% B in 45 min, 35 min hold at 99% B, 99—1% B in 5 min, 16 min re-equilibration (~11 column volumes). During the initial 8 min of isocratic elution, the sample loop was switched into flow, and the full loop volume (1.57 μL) was injected to D1.

Second Dimension Gradient Programming. In an earlier work on a unidimensional micro-LC system using an identically constructed binary gradient pump, we found that 30 s linear gradients can be performed with high accuracy and repeatability.[44] However, a pump-system dependent restriction is that fast gradients must not start or end at 0% of one of the eluent channels but at about 3—5% to ensure correct mixing and to prevent the eluent of one pump channel from entering the other. Therefore, in the second dimension, a water + 0.1% FA (eluent A)—acetonitrile + 0.1% FA (eluent B) gradient at a flow rate of 40 μL min^{-1} was programmed as follows: 3—97% B in 0.5 min, 0.1 min hold at 97% B, 97—3% B in 0.1 min, and 0.3 min re-equilibration at 3% B (~6.8 column volumes). The gradient delay was 0.025 min. Overall, each gradient cycle took 1 min. As the D2 cycle time usually equals the time for D1 fraction collection, the resulting transfer volume to D2 was 200 nL. As a consequence of a necessary pump refill, however, the transfer volume could not be held constant over the whole analysis time.

Modulation at Limited D2 Pump Volume. The used pneumatic pumps work like syringe pumps and, therefore, have a limited volume specified as 600 μL per gradient pump (300

μL per channel). We found that 13 consecutive D2 cycles could be run without interruption by an automatic pump restroke. The subsequent pump-stop, refill, and restart including flow and temperature stabilization at 3% B took 31 s on average (±1 s, standard deviation per 2D analysis: 0.6 s). Then, the next 13 D2 cycles were run and so forth.

One option was to collect 303 nL in each 13th D1 fraction (1 min + 31 s of refill time). In order to avoid a solvent strength effect which might significantly affect the analyte's peak shape and retention time in D2,[45,46] it was instead decided to split the larger fraction in two parts. During flow stabilization at 3% B, the last fraction collected prior to each pump refill was flushed to the D2 column. Although most analytes are trapped, unretained analytes are potentially lost—if the overall signal intensity is so low that no data analysis is possible in adjacent fractions—as they are flushed to the detector that is not acquiring data during the pump refill procedure. Subsequent to the next modulation valve switching, the remaining 103 nL that is collected during the 31 s of the pump refill procedure was transferred to D2 by the first of the next 13 D2 gradient cycles. By using the split transfer, 303 nL—in the following counted as one D1 fraction—could be analyzed in one D2 gradient cycle. The according modulation valve actuation was achieved by setting switching events in the gradient table of the repeated D2 pump method every full minute from 0 to 13 min. The described D1 fractionation is visualized in Supporting Information Figure S-1. The dependence of D1 retention time to the D1 fraction numbers is given in Table 1.

Table 1. Relation between D1 Fractions and D1 Retention Times

D1 fraction	collection time (min:s)	D2 gradient cycle	D1 retention time (min)
1−12	1:00	2−13	0−12
13	1:31	14	12−13.52
14−25	1:00	15−26	13.52−25.52
26	1:31	27	25.52−27.03
27−38	1:00	28−39	27.03−39.03
39	1:31	40	39.03−40.55
40−51	1:00	41−52	40.55−52.55
52	1:31	53	52.55−54.07
53−64	1:00	54−65	54.07−66.07
65	1:31	66	66.07−67.58
66−77	1:00	67−78	67.58−79.58
78	1:31	79	79.58−81.10
79−90	1:00	80−91	81.10−93.10
91	1:31	92	93.10−94.62
92−103	1:00	93−104	94.62−106.62

MS Parameters. Information-dependent acquisition (IDA)[47,48] was chosen as the MS strategy for a suspected screening, making use of both the single HRMS and the MS/MS functionalities of the QqTOF detector. In the primary experiments, "survey TOF scans" were carried out for an m/z range of 65−1000 in positive ESI ionization mode. Per IDA cycle (i.e., every 150 ms), the four most intensive precursor ions were chosen for sequential MS/MS scans with an m/z range of 50−1000 in order to obtain structural information from the respective product ion spectra. Based on the IDA cycle length, the programmed data acquisition rate was ∼6.7 Hz. The following ion source parameters were held constant during detection: the curtain gas was set to 10 psi, ion source gas 1 and 2 to 25 psi, IonSpray voltage floating to 5500 V, declustering potential to 80 V, and temperature to 500 °C. The collision energy was 10 and 40 eV for the survey TOF scan and the product ion scans, respectively.

Preparation of Contour Plots. Contour plots were generated in the following way: The corresponding MS chromatograms were exported to ASCII files, each containing ∼5200−5800 data points for 13 consecutive D2 gradient runs. Consequently, the real data acquisition rate was not constant and ranged from ∼6.7 to 7.4 Hz. To achieve a constant data point number, linear interpolation was performed in Excel 2010 (Microsoft, Redmont, WA) to 49 998 data points per file. Each file was then cut to achieve one chromatogram per D2 gradient run (i.e., 3846 points per chromatogram). Visualizations were prepared using OriginPro 9 by OriginLab (Northampton, MA).

RESULTS AND DISCUSSION

Evaluation of System Performance. At first, the 99-component mix was measured using the development system to gather LC × LC retention information on all of the 99 targets. Figure 2a shows a colored contour plot of the corresponding total ion current (TIC)-chromatogram.

As expected from a TIC, this perspective offers only limited information on the exact location of the analytes. Nonetheless, the high accuracy of the D2 gradient pump at the short cycle of 1 min can be seen from the noise lines at about 0.55, 0.59, and 0.78 min D2 retention time. The first two are observed at the end of the gradient, and the third occurs at the start of re-equilibration phase. Only for the signals of one series of 13 gradient cycles (compare the section Modulation at Limited D2 Pump Volume), a constant shift of about 0.01 min (0.6 s) to higher D2 retention is visible in Figure 2a. This is not an inaccuracy of the pump but a timer asynchronism caused by the use of two different computers and softwares for controlling the LC and the MS device. Thus, MS acquisition starts a bit too early for the shifted data.

The precise location of the analyte peaks in the 2D chromatogram was determined from extracted ion chromatograms (XIC). The monoisotopic mass of a target's protonated molecular ion $[M + H]^+$ ± 0.005 Da was chosen as the extraction window. In most cases, accurate mass verification allowed unambiguous identification of the target ions. Where necessary, the IDA MS/MS fragmentation pattern was additionally used. A detailed list containing the retention data of all targets is presented in Supporting Information Table S-2. Figure 2c contains an analyte map providing the locations of the peak maxima for every target after LC × LC separation of the 99-component standard mix. Peak width is not accounted for in this view and, therefore, separate spots do not necessarily mean baseline-separated compounds. Nonetheless, more than 90% of the targets resulted in countable spots that are spread over the chromatographic area. The results clearly underline that there is a significant difference in the selectivities of porous graphitic carbon (PGC) and C18 reversed-phase stationary phases, as is well-known from literature.[49] The additional selectivity helps to avoid coelution of isobars, as illustrated by the example of ifosfamide and cyclophosphamide in Supporting Information Figure S-2.

Surface coverage is often used as a measure of orthogonality in LC × LC.[50] Therefore, the ratio between the convex hull[51,52] that includes all analytes and the rectangle given by the minimum and maximum retention values in both dimensions was calculated as an approximation and comparative value. The

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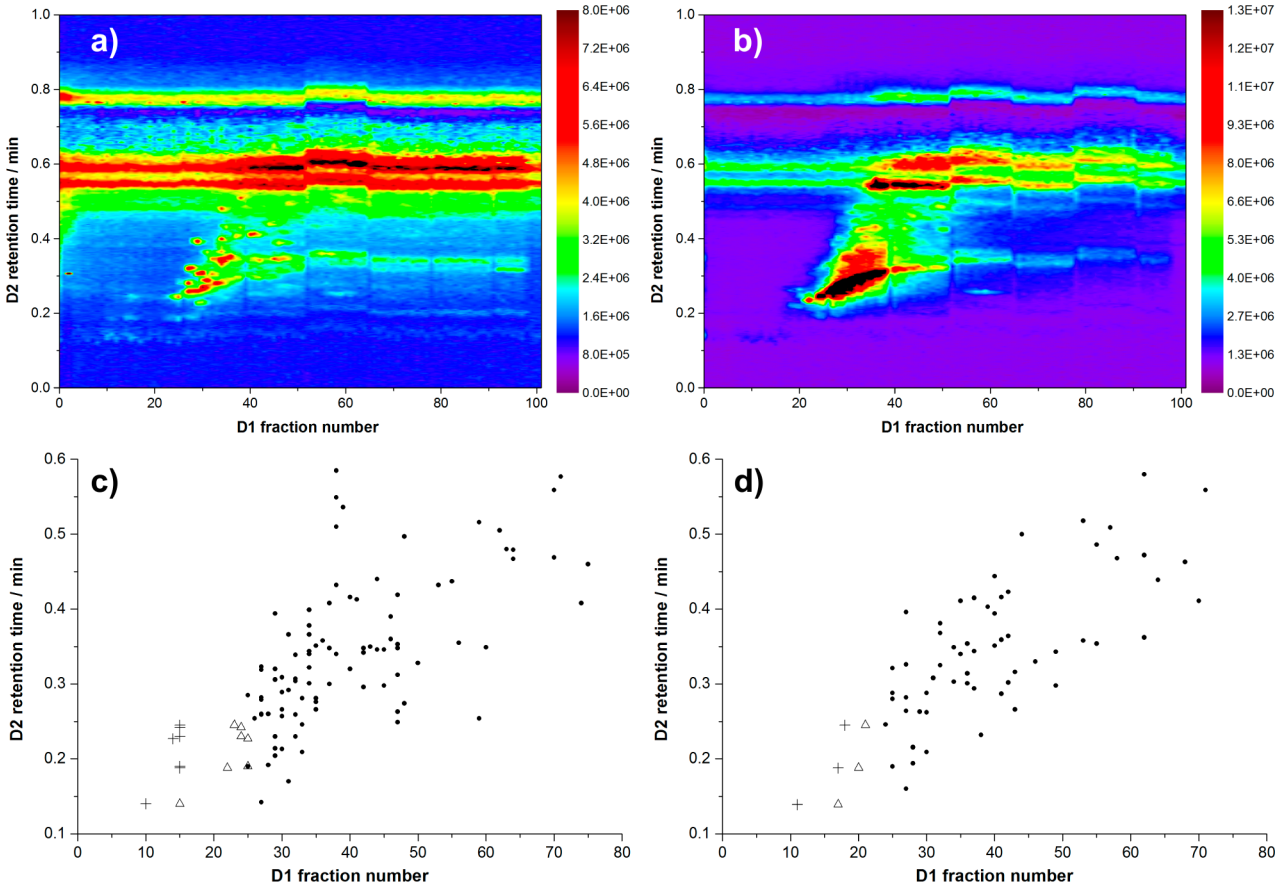

Figure 2. (a) and (b) Contour plots of the TIC chromatograms showing the LC × LC separations of a 99-component standard mix and a wastewater sample, respectively. Color code: signal intensity in counts s^{-1}. Black: intensities above maximum of color code. D1: Hypercarb 50 mm × 0.1 mm i.d., 5 μm. Mobile phase: water + 0.1% FA (A)−methanol + 0.1% FA (B) at 200 nL min^{-1}. D2: SunShell C18 50 mm × 0.3 mm i.d., 2.6 μm. Mobile phase: water + 0.1% FA (A)−acetonitrile + 0.1% FA (B) at 40 μL min^{-1}. Both dimensions operated in solvent gradient mode at 60 °C. D2 gradient cycle length: 1 min. Detection: TripleTOF 5600, TOF survey scan in positive ESI mode. Injection volume: 1.57 μL. For further details, refer to Materials and Methods. (c) and (d) Analyte maps for the standard mix and the targets in the wastewater sample, respectively, based on the analytes' peak maxima presented as ●. D1 double peaks are highlighted by + and Δ for first and second maximum, respectively (see the section Two-Dimensional Peak Shapes for further discussion).

area of the convex hull, an eight-sided irregular polygon, was determined using a vector-based method described by Dück et al.[53] This resulted in a surface coverage of 0.61 (or 61%). For more details, see Supporting Information page S-12.

Measurement of the Wastewater Sample. The TIC chromatogram of the online LC × LC separation of the wastewater sample is presented in Figure 2b. As overall signal intensities drastically increased compared to the standard, the influence of noise signals decreased. The highly populated area described by D1 fraction numbers 20−40 and D2 retention times 0.2−0.4 highlights the importance of using a second dimension to separate the solutes in this type of water sample. Looking at the high signal intensities in this area, the application of only one of the dimensions would result in significantly increased coelutions and stronger suppression of analyte ionization by ionizable matrix.

Using the results of the 99-component standard mix, a suspected screening was performed for the wastewater sample. Sixty-five of the 99 suspected targets and, additionally, all three deuterated internal standards were found in the sample (see Supporting Information Table S-3 for details).

Two-Dimensional Peak Shapes. In Figure 3, the XIC chromatograms of 10 analytes in the standard mix were summed. These analytes represent different (border) regions of

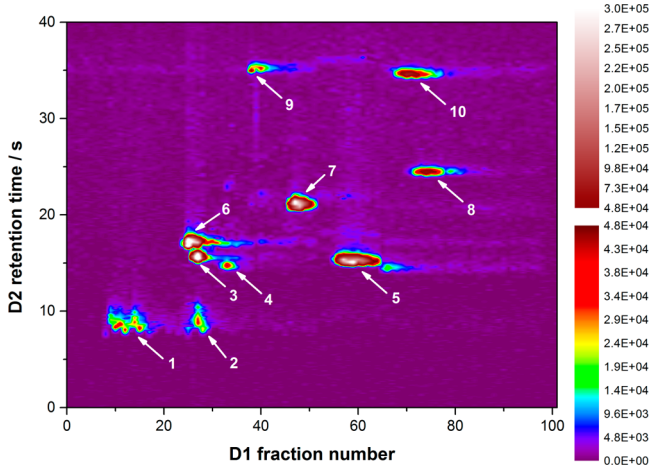

Figure 3. Chromatogram of summed XICs for 10 substances. Sample: 99-component standard mix. Color codes: signal intensity in counts s^{-1}. White: intensities from 3.0×10^5 to 4.7×10^5 counts s^{-1}. Analytes: (1) metformin, (2) gemcitabine, (3) tramadol, (4) sulfapyridine, (5) pipamperone, (6) venlafaxine, (7) clarithromycin, (8) diuron, (9) HT-2 toxin, (10) fenofibrate. Extracted m/z: protonated molecular ion $[M + H]^+ \pm 0.005$.

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the analyte map (Figure 2c). This way, Figure 3 can be used to give an overview on the different types and extremes of peak shapes that were observed using the presented method.

An extremely distorted peak shape is observed for metformin (1). It was the only analyte of the mixture for which peak focusing could not be established on any of the columns. The very low retention of metformin was severely disturbed by the injected solvent and the temperature conditions that were applied. Missing first dimension focusing (~fractions 10 to 25) is marked in the analyte maps by spots for both D1 peak maxima (Figure 2c,d). Although first dimension focusing was established for gemcitabine (2), second dimension focusing was still missing. All peaks apart from that of metformin and gemcitabine were focused in D2. Thus, the border for D2 focusing was at about 10 s (or 0.17 min) D2 retention time, which is still very close to D2 dead time (~0.09 min). The symmetrical peak shape of sulfapyridine (4) is an example for those of many peaks that eluted in the impact range of both gradients. The mass concentration of the targets in the standard mix had been chosen very high (1 μg mL^{-1}) to ensure detection of all analytes. This, however, leads in several cases to a peak broadening and, depending on the retention behavior of the analytes, results in a more or less pronounced tailing as in case of tramadol (3), venlafaxine (6), and clarithromycin (7). For those analytes eluting in D1 hold time at a maximum % B, as in case of pipamperone (5), diuron (8), and fenofibrate (10), strong tailing was observed.

Looking closely to the peaks in Figure 3, especially those with tailing, a slightly serpentine form of the peaks in the direction of D1 is visible instead of a straight orientation. This effect (~0.002 min D2 retention deviation) is caused by the fact that one of the two modulation loops is emptied in the opposite flow direction. The effect of the enlarged fractions can be seen best for analytes 5, 8, and 9. The stepwise injection of the enlarged fraction leads to peak broadening and therefore to an intensity collapse for this fraction. Thus, peaks seem to be cut in two parts. For pipamperone, this effect is mixed with the retention shift due to the timer asynchronism (see the section Evaluation of System Performance, compare Figure 2a).

Overall, the lowest observable D1 width for identifiable peaks was three to four fractions. This was also true for the analytes in the wastewater sample, so the M−S−F criterion was fulfilled for all identified compounds. Identification of analytes eluting in the region of the enlarged fractions was not hampered as these analytes were found in adjacent fractions as well.

Performance of the LC Dimensions. *First Dimension.* The decision to select Hypercarb in D1 was not solely based on orthogonality criteria. As demonstrated by Stoll et al., who used a carbon-clad zirconia phase in a high-temperature second dimension,[34] carbon stationary phases provide extraordinary retentivity and peak focusing abilities, even at high temperatures and disadvantageous sample solvent conditions. These properties of the Hypercarb phase were needed to implement the large volume injection at elevated temperatures, especially as samples containing a stronger solvent (5% acetonitrile) than the initial mobile phase (3% methanol) were injected. As can be seen from Figures 2c,d and 3, peak splitting could be successfully avoided for the majority of targets. Although the high retentivity is beneficial for the large volume injection, it extends retention time of late-eluting compounds (Figure 3).

Stability of the D1 retention times between wastewater and standard mix (mean of absolute fraction deviations, 3.4; standard deviation, 2.6) makes it clear that for future quantification methods and a proper identification via D1 retention time, standard addition approaches will be essential. The high load of sample matrix due to the large volume injection as well as the high analyte concentrations in the standard mix surely contributed to the observed deviations. However, retention data of the internal standards and, where available, the IDA-MS/MS experiments could be used to unambiguously identify the targeted wastewater contaminants.

Second Dimension. As can be seen from Figure 4, the SunShell C18 column in D2 provided sharp, symmetrical peak shapes for almost all analytes at high transfer volume conditions and comparably high solute concentration.

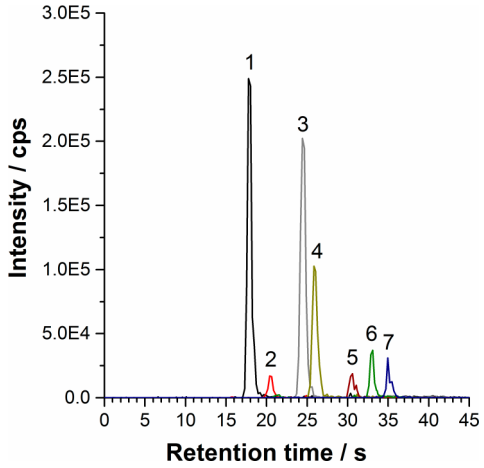

Figure 4. Second dimension XIC chromatogram overlay for seven analytes in D1 fraction 38 of the standard mixture. Analytes: (1) 5-methyl-1*H*-benzotriazole, (2) prednisolone, (3) isoproturon, (4) stachybotrylactam, (5) picoxystrobin, (6) simvastatin, and (7) HT-2 toxin. Extracted *m*/*z*: protonated molecular ion $[M + H]^+ \pm 0.0005$.

The high performance of D2 was underlined by very stable retention times. The mean of the absolute D2 retention time deviations between the targets in the water sample and standard (compare Table S-2) was 0.003 min (standard deviation: 0.003 min). This makes it clear that matrix effects that affect first dimension retention are absent in the second dimension. A similar retention time stability of D2 was observed within one sample (i.e., regarding the differences between D1 fractions). The average peak width was 2 s. Although faster gradients may be performed, the QqTOF-MS acquisition rate dominated by the already optimized IDA cycles would not allow a sufficient number of data points for significantly narrower peaks.

Sensitivity of the System. The same wastewater sample had been analyzed on conventional 1D-LC-MS/MS systems with triple quadrupole detection using validated methods. The analyte list of these measurements contained 56 of the 99 targets. These were quantified using multiple reaction monitoring methods. The results listed in Supporting Information Table S-3 allow a rough estimation of the detection performance of the multidimensional system. Environmentally relevant analyte concentrations in the lower ng mL^{-1} range or even below—depending on the analyte—were detected. This is a good starting point for further optimizations toward the ng L^{-1} range. However, these supplemental values cannot replace future in-depth sensitivity validations of methods developed on the basis of the multidimensional system.

Solvent Consumption. The overall D2 solvent consumption of the presented miniaturized online LC × LC was compared to that of conventional fast D2 approaches that use flow rates between 1 and 5 mL min^{-1}. While conventional fast D2 systems consume 0.6 to 3 L of solvent over 10 h, only 0.024 L are needed by a miniaturized D2 system operated at 40 μL min^{-1}. Keeping the rising solvent costs in mind, this trivial comparison underlines the economic advantage of miniaturized, online LC × LC systems over the nonminiaturized versions.

CONCLUSIONS AND OUTLOOK

The presented multidimensional system provides the opportunity to use the advantages of online LC × LC and hybrid detection for the screening analysis of complex samples. As the LC × LC is consistently miniaturized, a fast operation of the second LC dimension is possible at low flow rates. Consequently, the hyphenated systems work in full compatibility without the need for a flow split. The capabilities of the highly customizable system were demonstrated by a suspected screening of 99 targets in a wastewater sample. PGC employed in the first and core−shell C18 in the second dimension provided a good LC × LC performance for the separation of the wastewater contaminants (convex hull surface coverage: ∼0.6). D2 retention time turned out to be a reliable additional criterion for the identification of suspected compounds, as its stability was very high. The overall splitless design of the capillary system in combination with the large volume injection in the first LC dimension allowed countervailing the sensitivity losses due to modulation and resulted in sufficiently high sensitivity to detect the contaminants at environmentally relevant concentrations. The overall low solvent consumption of the system makes the multidimensional system an economically attractive and environmentally friendly alternative to existing systems based on online LC × LC approaches that use conventionally sized D2 columns. For future use, separate heating of both columns could be beneficial for an additional optimization of the first dimension toward analyte focusing at very low and very high retention times. A continuous-flow pump in the second dimension should be considered to simplify system programming and data analysis by making the refill procedure obsolete. For future quantification experiments, a continuous flow delivery is a must as only constant D1 sampling and constant transfer to D2 ensure true (quantitative) comprehensiveness of the multidimensional system.[54] The existing screening approach should already allow an extension of the data analysis to suspected and unknown analytes without reference material.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

1D-LC, one-dimensional liquid chromatography; APCI, atmospheric pressure chemical ionization; capLC, capillary liquid chromatography; D1, first dimension; D2, second dimension; ESI, electrospray ionization; FA, formic acid; HRMS, high-resolution mass spectrometry; IDA, information-dependent acquisition; IEC, ion exchange chromatography; LC × LC, comprehensive two-dimensional liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; *m*/*z*, mass-to-charge ratio; MS, mass spectrometry; MS/MS, tandem mass spectrometry; M−S−F, Murphy−Schure−Foley; nanoLC, nano liquid chromatography; PGC, porous graphitic carbon; QqTOF, quadrupole/time-of-flight (mass spectrometer type); SPE, solid-phase extraction; TIC, total ion current; TOF, time-of-flight; UHPLC, ultrahigh-performance liquid chromatography; v, volume; XIC, extracted ion chromatogram

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