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Compound Identification Using Liquid Chromatography and High-Resolution Noncontact Fraction Collection with a Solenoid Valve

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Abstract

We describe the development of a high-resolution, noncontact fraction collector for liquid chromatography (LC) separations, allowing high-resolution fractionation in high-density well plates. The device is based on a low-dead-volume solenoid valve operated at 1–30 Hz for accurate collection of fractions of equal volume. The solenoid valve was implemented in a modified autosampler resulting in the so-called FractioMate fractionator. The influence of the solenoid supply voltage on solvent release was determined and the effect of the frequency, flow rate, and mobile phase composition was studied. For this purpose, droplet release was visually assessed for a wide range of frequencies and flow rates, followed by quantitative evaluation of a selection of promising settings for highly accurate, repeatable, and stable fraction collection. The potential of the new fraction collector for LC-based bioactivity screening was demonstrated by fractionating the LC eluent of a mixture of estrogenic and androgenic compounds, and a surface water sample (blank and spiked with bioactives) combining mass spectrometric detection and two reporter gene assays for bioactivity detection of the fractions. Additionally, a mixture of two compounds was repeatedly LC separated and fractionated to assess the feasibility of the system for analyte isolation followed by nuclear magnetic resonance analysis.

Keywords

liquid chromatography, fraction collection, solenoid valve, effect-directed analysis, reporter gene assay, NMR

Introduction

Fraction collection of liquid chromatography (LC) separations can be utilized for analyte purification, as well as for applications in which a detection technique cannot be coupled online to LC due to compatibility issues. Fractionation is commonly applied in environmental toxicant screening,[1–8] drug discovery research,[9–14] and food chemistry[15–18] when chemical analysis is combined with bioassay testing for the identification of bioactive substances. In these types of studies, fractionation is required to reduce the sample complexity until only one compound is present in a fraction. Subsequently, each fraction is tested for biological activity and the detected bioactives are potentially identified. Fractions of LC eluates have also been collected on matrix-assisted laser desorption ionization (MALDI) target plates[19,20] for analyte identification by mass spectrometry (MS). Especially for proteomics, fraction collection of

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nano-LC separation on MALDI plates has been used for peptide characterization. In another approach, fractions of nano-LC separations have been collected in pipette tips and dried at room temperature, and subsequently reconstituted for chip-based direct-infusion MS.[21] By disconnecting the LC separation from MS detection, different reconstitution solvents can be selected for optimal analyte ionization. Fraction collection of LC separations also finds use in analyte isolation and enrichment in many research areas for offline analysis by nuclear magnetic resonance (NMR) spectroscopy.[17,18,22–24] For this purpose, fractions are normally collected in vessels followed by lyophilization and reconstitution in an NMR-suitable solvent.[24]

Fraction collection approaches in LC vary significantly among the research fields. In many studies, relatively large fractions, corresponding to 1 to several minutes, are collected, which obviously results in loss of chromatographic resolution.[1,4,17,25] Consequently, repeated fractionation cycles are required for analyte isolation, which is time-consuming and prone to analyte losses. In environmental chemistry employing effect-directed analysis (EDA), recently the approach has been shifting toward the collection of small-volume fractions corresponding to 2–10 s of LC time.[26–28] This approach has already been shown feasible and useful in the field of drug discovery.[11–13,29]

To achieve the collection of discrete volumes, in the vast majority of existing fraction collectors droplets of LC effluent are released from a capillary tip by gravitational force and/or are deposited by allowing the tip to contact the collection surface using robotics providing *x*-*y*-*z* movements. In another approach, the tip is moved slightly above the surface of the collection target for droplet deposition by liquid contact. The latter is mostly used for narrow-bore LC separations or when the collection device can hold small volumes only (e.g., a 1536-well plate). Without active deposition/release, LC droplets would adhere to the tip and droplet sizes may exceed the well volume, causing overflowing toward surrounding wells. Additional movement in the *z* direction limits the speed at which the tip can be moved between the respective deposition spots. For standard-bore LC separation systems, droplet deposition by contact of the effluent with the target surface is less common, but the droplet release rate can be insufficient when small fractions ($<$10 s) have to be collected, resulting in varying fraction volumes. Kool et al. developed a fraction collector in an attempt to overcome these issues.[10] The device was based on a small metal cylinder hitting a section of flexible tubing to force droplet ejection, and its performance was demonstrated for flow rates ranging from nL/min up to 250 µL/min. However, the instrument mechanics demanded regular maintenance and replacement of the flexible tubing to ensure acceptable reproducibility. Moreover, when operated at high frequencies, which were required for handling increased flow rates, the device suffered from overheating.

In this study, we describe the development of a new fraction collector capable of high-frequency droplet ejection allowing contact-free accurate fraction collection of standard-bore LC separations, while accommodating a wide range of LC flow rates. To this end, a solenoid valve was incorporated in an autosampler device and instrumental modifications together with the development of a software script for device control were made and tested. To the best of our knowledge, the use of a solenoid valve for fraction collection of LC separations has not been reported before. The solenoid valve supply voltage and pulse width for droplet ejection were optimized, and an external electronic control unit was constructed to provide optimal voltage and enable accurate adjustment of the pulse width. The device performance was initially assessed by visual inspection of the droplet release for different flow rates, frequencies, and solvent viscosities. The stability was investigated by continuous supply and fraction collection of a fluorescent dye solution followed by plate reader analysis. LC fraction collection, detection, repeatability, and resolution were studied by fractionating a fluorescent dye as well as a dye mixture with online detection, permitting comparison with the reconstructed signal from fraction collection. The potential of the new device for LC bioactivity screening was evaluated by fractionating the LC separation of a surface water sample and a mixture containing estrogenic and androgenic compounds. Two reporter gene assays were used for bioassay detection, while chemical detection by MS was performed in parallel.

Materials and Methods

Chemicals and Materials

Methanol (MeOH), acetonitrile (ACN), and formic acid (FA), all ultra-high-performance liquid chromatography–mass spectrometry (UHPLC-MS) grade, were purchased from Biosolve B.V. (Valkenswaard, Netherlands). Quinine hydrochloride dihydrate, acetic acid, diammonium phosphate, and the colorants New Coccine and Brilliant Blue were obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). Sodium acetate trihydrate was from J.T.Baker (Deventer, Netherlands). Well plates (white, flat-bottom, 384-well plates; white, flat-bottom, 384-well plates with a transparent bottom; and black, flat-bottom, 384-well plates) were obtained from Greiner Bio-One B.V. (Alphen aan den Rijn, Netherlands). The following reagents were used for bioassay testing. VM7Luc4E2 cells were kindly provided by Michael Denison (University of California, Davis, CA) to screen for estrogenic activity. The AR-Ecoscreen assay was used for the detection of androgenic compounds. Dulbecco's modified Eagle medium (DMEM) containing F12 GlutaMAX, low glucose, phenol-free DMEM, and DMEM/F12 L-glutamine were obtained from Thermo

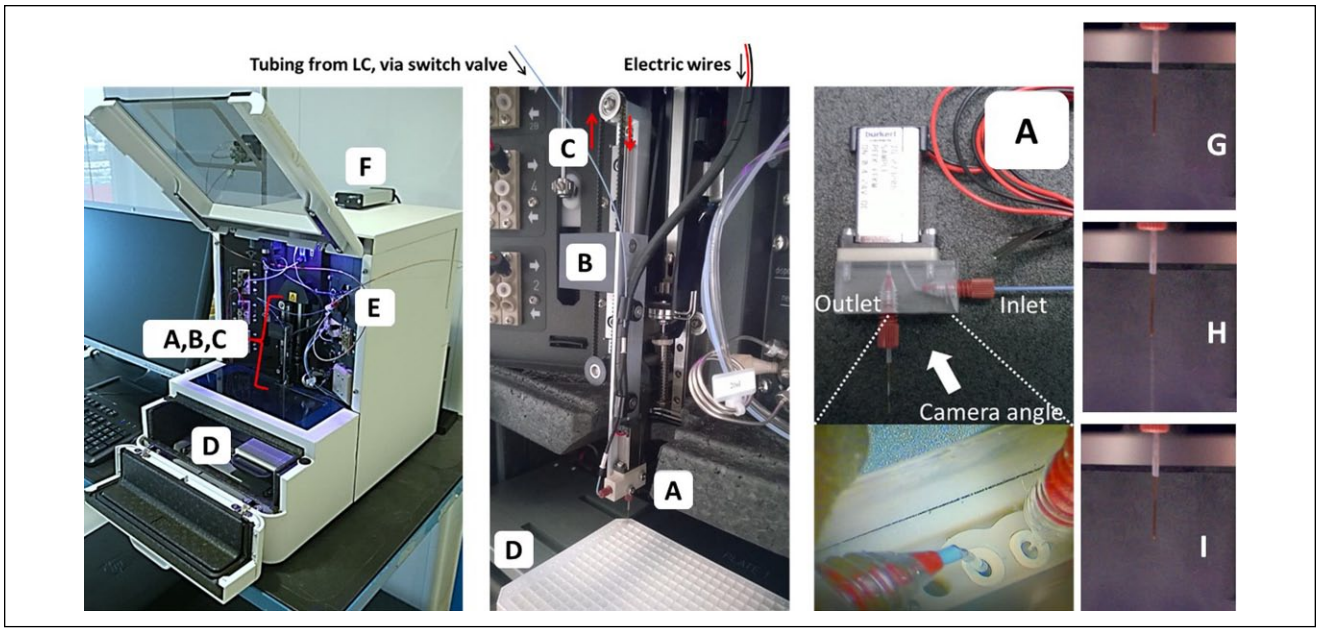

Figure 1. Solenoid valve-based fraction collector. (**A**) Solenoid valve with connection piece enabling connection with LC tubing (inlet) and a deactivated fused-silica capillary (outlet). (**B**) Assembly connecting the stainless steel mounting piece for the solenoid valve with the string for height adjustment of the solenoid valve. (**C**) Rubber cord enabling height adjustment of the solenoid valve above the well plate. (**D**) A 384-well plate. (**E**) Switch valve. (**F**) Electronic signal converter. (**G**) Solenoid valve closed. (**H**) Solenoid valve open; ejection of solvent in a narrow beam. (**I**) Solenoid valve closed.

Fisher (Landsmeer, Netherlands). Penicillin, streptomycin, charcoal-stripped fetal bovine serum (FBS), DMSO, D-luciferin, tris(hydroxymethyl)aminomethane (TRIS), dithiotreitol (DTT), glycerol, Triton-X100, geneticin (G418), phosphate-buffered saline (PBS), adenosine triphosphate (ATP), 1,2-cyclohexylenedinitrilo-tetraacetic acid (CDTA), coenzyme A, hygromycine, zeocin, ethylenediaminetetraacetic acid (EDTA), estriol (E3), β-estradiol (β-E2), ethynylestradiol (EE2), bisphenol A (BPA), androstenedione, and testosterone were obtained from Sigma-Aldrich.

Fraction Collector

Fraction collection was performed using a FractioMate prototype (Spark Holland, Emmen, Netherlands; Vrije Universiteit, Amsterdam, Netherlands). This solenoid valve-based fraction collector was built by adding a noncontact spotter unit to a standard Integrity autosampler from Spark Holland. A Bürkert type 6712-2/2-way whisper valve (Bürkert, Breda, Netherlands) was mounted for the high-frequency droplet generation and as such operated as the noncontact spotter part for high-frequency droplet generation. **Figure 1A** shows the device, highlighting the location of the solenoid valve connected to 1/32-inch LC tubing (inlet) and a deactivated fused-silica capillary of 3 cm in length and 250 μm i.d. (outlet). Two white O-rings ensured a leak-tight connection between the solenoid valve and the connection piece. The final version of the connection piece was made of polyetheretherketone (PEEK), providing material robustness and chemical resistance against organic solvents. The solenoid valve was mounted on a stainless steel unit that also guided the electric wires and solvent tubing toward the solenoid valve. The stainless steel mounting unit was connected to an assembly (**Fig. 1B**) that gripped one side of the cord (**Fig. 1C**), which could be moved vertically to allow adjustment of the space between the solenoid valve and the well plate (**Fig. 1D**). The switch valve (**Fig. 1E**) was used to direct the LC flow to either the waste or the solenoid valve. An external electronic signal converter (**Fig. 1F**) was developed for accurate control of the solenoid valve in terms of frequency and pulse width selection supplied to the solenoid valve, and for allowing an operational voltage of up to 28 V for solvent ejection. Modifications to the device were performed such that the original autosampler function was maintained.

Solenoid Valve Operating Voltage and Pulse Width

The standard operating voltage of the solenoid valve was 24 V, and the pulse width could be adjusted with the developed software script controlling the output signal of an unused port on the electronic circuit of the autosampler. To test the

influence of the supply voltage on the solenoid valve and to reduce the step size in which the pulse width could be regulated, an electronic signal converter was developed, providing the solenoid valve with 28 V and enabling the selection of pulse widths ranging from 2 to 11 ms in steps of 1 ms.

Droplet Formation at Different Flow Rates, Frequencies, and Solvent Viscosities

The relation between the flow rate and solenoid valve frequency, as well as the influence of the solvent viscosity, was investigated by fractionating different solvent mixtures. The following flow rates were tested: 0.1, 0.3, 0.6, and 1.0 mL/min. For each flow rate, frequencies from 1 to 10 Hz (steps of 1 Hz) and 15, 20, 30, and 50 Hz were tested. This was performed for each of the following solvent compositions reflecting different stages of commonly used LC gradients: water/MeOH 98/2 (v/v), water/MeOH 50/50 (v/v), water/MeOH 2/98 (v/v), water/ACN 98/2 (v/v), water/ACN 50/50 (v/v), and water/ACN 2/98 (v/v). These solvent compositions were continuously supplied to the solenoid valve by an LC pump.

Fraction Collection Stability

The two most promising frequencies for each flow rate tested from the droplet formation experiment (visual assessment) were tested quantitatively. The stability of the fraction collection process was studied by collecting fractions of a continuously supplied fluorescent solution in 384-well plates that contained 100 µL of 0.1 M sodium acetate buffer (pH 5) per well. To this end, a 200 mL superloop filled with the fluorescent solution was placed in between the LC pump and solenoid valve. For each experiment, 120 fractions were collected consecutively at a constant solenoid frequency. Subsequently, the fluorescence signal (λ_{ex} 330 nm, λ_{em} 380 nm) of each fraction was measured with a Thermo VarioSkan plate reader (Thermo, Breda, Netherlands). Six different combinations of commonly used LC solvents containing 1 mM quinine were prepared to mimic the viscosity at the initial, intermediate, and final stage of a generic LC gradient (water/MeOH 98/2, water/MeOH 50/50, water/MeOH 2/98, water/ACN 98/2, water/ACN 50/50, and water/ACN 2/98 [v/v]). A superloop (a cylinder with a floating piston filled with sample or other material, such as reagents; the eluent forces the piston forward, ejecting the sample/reagents) filled with the test solution was connected in between a Shimadzu LC20AD pump (Shimadzu, Breda, Netherlands) operated at 0.1, 0.3, 0.6, or 1.0 mL/min and the fraction collector.

Sample Fractionation and Detection, Resolution, and Repeatability Assessment

To study fraction collection resolution, injected quinine was fractionated after LC chromatography (50, 100, and 150 µM) in triplicate using a Waters XBridge BEH C18 column (1.5 µm; 4.6 × 100 mm; Etten-Leur, Netherlands), isocratic elution with MeOH/ACN/0.1 M acetic acid (pH 5) (45/15/40 [v/v]) at a flow of 0.4 mL/min (10 µL injection), and a Shimadzu RF-10axl fluorescence detector. Fractions were collected in 384-well plates filled with 100 µL of acetic acid/acetate buffer, pH 5 (0.1 M), at a solenoid valve frequency of 10 Hz with a 5 s fraction interval time. Plate reader analysis and online fluorescence detection were performed at λ_{ex} 330 nm and λ_{em} 380 nm.

A solution containing 200 µg/mL New Coccine (red color) and 70 µg/mL Brilliant Blue G (blue color) was separated on a Waters XBridge BEH C18 column (3.5 µm; 2.1 × 50 mm; using a flow rate of 0.5 mL/min); mobile phase A was water/ACN 98/2 (v/v) and solvent B was water/ACN 50/50 (v/v). Both A and B contained 20 mM diammonium phosphate buffer (pH 8).[30] The gradient started at 10% B, which was held constant for 3 min and subsequently increased to 95% within 4 min and maintained at this composition for another 5 min. Next, the percentage of B was returned to 10% and held constant for 5 min. The column effluent was directed via a Shimadzu SPD20AV UV/VIS detector to the fraction collector. The fraction interval time was set at 3.5 s and the solenoid valve frequency was 10 Hz. Fraction collection was started 5 min after sample injection. The LC analysis was performed in triplicate and fractions were collected in white 384-well plates with a transparent bottom for plate reader absorbance detection. Brilliant Blue was detected at a wavelength of 595 nm and New Coccine at 500 nm.

Bioactivity Screening

A solution containing 2 nM β-E2, 20 nM E3, 10 nM testosterone, 10 nM androstenedione, and 400 nM BPA was prepared. Blank surface water and surface water spiked with β-E2 (both 0.5 L) were extracted in duplicate following a published procedure.[27] The theoretical concentration of β-E2 reached after extraction of the spiked water extract was estimated at 2.6 nM. LC analysis was performed on a Thermo Scientific Dionex UltiMate 3000 ultra-high-performance LC system (Thermo Scientific, Amsterdam, Netherlands) equipped with a Waters ACQUITY UPLC BEH C18 Column (130 Å, 1.7 µm, 2.1 × 150 mm). Milli-Q water and MeOH were used for LC separation at a flow of 0.4 mL/min and 55 °C starting at 1% MeOH (v/v), followed by a linear gradient of 20 min to 99% MeOH (v/v), which was held constant for 2 min, after which the MeOH percentage was returned to initial conditions. The injection volume was 250 µL. Using a split, 10% of the column effluent was split toward a Bruker Impact II time-of-flight mass spectrometer with an electrospray ionization (ESI) source and 90% toward the fraction collector. Samples were analyzed in positive and negative mode over the *m*/*z* range 30–1000

in MS-MS/MS mode. The source parameters were as follows: end plate offset, 500 V for both polarities; capillary positive mode, 2500 V; negative mode, 4500 V; nebulizer; and dry gas pressure, flow, and temperature, 2 bar, 8.0 L/min, and 200 °C, respectively, for both polarities. The mass analyzer settings for detection in positive mode were as follows: both funnels at 150 peak-to-peak voltage (Vpp); hexapole radiofrequency (RF), 30 Vpp; quadrupole ion energy, 6.0 eV; collision RF, 250–1000 Vpp; transfer time, 25–70 μs; collision energy for MS/MS, 20 eV; and prepulse storage, 5 μs. For the negative mode, the settings were as follows: both funnels at 200 Vpp; hexapole RF, 35 Vpp; quadrupole ion energy, 5.0 eV; collision RF, 250–1000 Vpp; transfer time, 25–65 μs; collision energy for MS/MS, 20 eV; and prepulse storage, 5 μs. Fractions of 6.5 s were collected in 384-well plates. The protocol of Rogers and Denison[31] was followed for cell culturing of the VM7Luc4E2 cells. The AR-Ecoscreen cells were cultured as described by Araki et al.[32] The 384-well plates were dried via vacuum centrifugation to remove organic solvent prior to bioassay analysis. VM7Luc4E2 cells were used for the detection of estrogenic compounds. AR-Ecoscreen cells were used for the detection of androgens and androgen-like compounds. Bioassay testing was performed as described before[27] with modifications (in 384-well plate format). In brief, cells were seeded at a concentration of 200,000 cells/mL in white, flat-bottom, 384-well plates with transparent bottom. DMEM, phenol-free low glucose, was used for VM7Luc4E2, and DMEM/F12, phenol-free L-glutamine, was used for AR-Ecoscreen cells. Each well was filled with 20 μL of the cell suspension and the outer two rows were filled with 100 μL of ultrapure water. The well plates containing seeded cells were incubated for 24 h at 37 °C and 5% CO_2. The following day, 5 μL of the reconstructed fractions was transferred to the cells using a multichannel pipette. For reconstitution a 50 μL volume of assay medium containing stripped FBS was added to the fractions for reconstitution and shaken for 10 min at 500 rpm. Cells were exposed for 24 h followed by visual inspection and lysed. A plate reader was used for bioassay readout and a chromatogram was constructed by plotting the bioassay response of each fraction against the corresponding fraction time. Comparison of the MS chromatogram with the bioassay trace allowed the pinpointing of bioactive peaks.

Fraction Collection for NMR Analysis

A mixture of EE2 and BPA (200 μM each; 50 μL injected) was injected on a Waters XBridge BEH C18 column (3.5 μm; 2.1 × 50 mm) at a flow rate of 0.4 mL/min using two Shimadzu LC20AD pumps and a Shimadzu UV detector. Solvents A and B consisted of water/MeOH/FA (98/2/0.1) and water/MeOH/FA (2/98/0.1), respectively. The gradient was as follows: 1 min at 50% B, increase to 100% B in 19 min, return to 50% B in 0.1 min, and equilibration at 50% B for 5 min. Absorbance detection was performed at 275 nM. Effluent fractions of 5 s were collected in a 384-well plate moving in a serpentine fashion over the well plate starting from well A1. The solenoid valve frequency was set at 10 Hz. Before starting the separation of the EE2 and BPA mixture, the delay between the UV detector and fraction collector was determined to ensure that the EE2 and BPA fraction times could be calculated from the UV elution time. To this end, a Brilliant Blue G solution was injected and the retention time measured by UV (595 nm) and the fraction time of the blue-colored fraction were compared. Next, the BPA/EE2 mixture was separated and fractionated in triplicate using a one-well plate with overlaid fractions. This procedure was repeated, yielding a total of 27 separations fractionated on nine-well plates (each plate containing three analyses). Plates were dried with a vacuum centrifuge. Fractions from plate 1 containing BPA were reconstituted and gathered in 700 μL of deuterated DMSO. The same was done for the fractions containing EE2. The fractions containing EE2 or BPA from plates 2–4 were pooled in a final volume of 700 μL. The same was performed for plates 5–9. From the obtained samples NMR spectra were recorded on a Bruker Avance 500 using the residual solvent peak as an internal standard (^{1}H: δ 2.50 ppm for DMSO-d_6). In detail, the following signal was obtained for BPA: ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.16 (s, 2H), 6.98 (d, 4H, J = 8.70 Hz), 6.64 (d, 4H, J = 8.70 Hz), and 1.53 (s, 6H). The spectra measured for the EE2-containing fractions was as follows: ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.01 (s, 1H), 7.06 (d, 1H, J = 8.45 Hz), 6.51 (dd, 1H, ^1J = 8.40 Hz, ^2J = 2.60 Hz), 6.44 (d, 1H, J = 2.50 Hz), 5.35 (s, 1H), 2.72–2.65 (m, 2H), 2.34–2.26 (m, 1H), 2.15–2.01 (m, 2H), 1.87 (td, 1H, ^1J = 12.72 Hz, ^2J = 3.47 Hz), 1.82–1.73 (m, 2H), 1.71–1.56 (m, 3H), and 1.37–1.20 (m, 4H), 0.76 (s, 3H).

Results and Discussion

A solenoid valve was implemented in an autosampler device to achieve high-frequency and reproducible droplet ejection in well plates over a wide range of LC flow rates. Necessary mechanical modifications were made to the autosampler device. An external electronic unit was constructed for accurate control of the solenoid valve and steering software was written. For the prototype, several parameters, such as supply voltage, pulse width, and frequency, were evaluated as a function of mobile phase flow rates and compositions.

Solenoid Valve Parameters

Different solenoid valve pulse widths were tested to assess the influence on droplet release. A pulse width of 4 ms was shown to be optimal for solenoid valve frequencies

Table 1. Assessment of Droplet Ejection Using Classification by Four Levels.

		Frequency (Hz)													
Solvent	Flow (mL/min)	1	2	3	4	5	6	7	8	9	10	15	20	30	50
Water/MeOH 98/2	0.1	1	1	1	1	1	1	1	1	1	1	1	1	4	4
	0.3	2	3	1	1	1	1	1	1	1	1	1	1	1	1
	0.6	4	2	2	2	2	1	1	1	1	1	1	1	1	1
	1.0	4	2	2	2	2	2	2	2	2	1	1	1	1	1
Water/MeOH 50/50	0.1	1	1	1	1	1	4	4	4	4	4	4	4	4	4
	0.3	4	2	2	1	1	1	1	1	1	1	1	4	4	4
	0.6	4	4	4	4	2	2	1	1	1	1	1	1	1	4
	1.0	4	4	4	4	4	4	4	4	4	2	1	1	1	1
Water/MeOH 2/98	0.1	1	1	1	1	1	1	2	2	2	2	2	2	2	4
	0.3	4	2	1	1	1	1	1	1	1	1	1	4	4	4
	0.6	4	4	4	2	1	1	1	1	1	1	1	1	1	2
	1.0	4	4	4	4	4	2	2	1	1	1	1	1	1	1
Water/ACN 98/2	0.1	1	1	1	1	1	1	1	1	1	1	1	1	4	4
	0.3	2	2	1	1	1	1	1	1	1	1	1	1	1	1
	0.6	2	2	2	2	2	1	1	1	1	1	1	1	1	1
	1.0	2	2	2	2	2	2	2	2	1	1	1	1	1	1
Water/ACN 50/50	0.1	1	1	1	1	1	1	4	4	4	4	4	4	4	4
	03	2	2	1	1	1	1	1	1	1	1	1	1	4	4
	0.6	4	4	4	4	2	1	1	1	1	1	1	1	1	4
	1.0	4	4	4	4	4	4	2	2	2	1	1	1	1	1
Water/ACN 2/98	0.1	1	1	1	1	2	4	4	4	4	4	4	4	4	4
	0.3	2	4	1	1	1	1	1	1	1	1	4	4	4	4
	0.6	4	4	4	2	1	1	1	1	1	1	1	1	4	4
	1.0	4	4	4	4	4	4	4	1	1	1	1	1	1	1

(1) Proper droplet ejection in a straight jet in the correct well in accordance with the applied solenoid valve frequency. (2) Droplet release by the solenoid valve frequency, but distorted ejection angle as a result of the continuous presence of the small droplet at the capillary tip. (3) Liquid is ejected as a spray. (4) Formation of a large drop at the tip of the exit capillary, followed by its eventual release due to gravitational force.

between 1 and 50 Hz employing LC flow rates ranging from 0.1 to 1.0 mL/min. Evaluation of different supply voltages revealed that at standard operating voltage (24 V), droplet release induced by the solenoid valve (not by gravitational pull) was only occurring at higher flow rates (>0.4 mL/min). A slight increase in supply voltage to 28 V allowed solenoid-induced droplet ejection over the entire range of flow rates. The higher voltage was accompanied by an increased loudness of the ticking sound generated by the solenoid valve during fraction collection, indicating an increased mechanical pulse strength, which aids droplet ejection.

Droplet Formation

Following adjustment of the solenoid supply voltage and pulse width, droplet ejection was visually evaluated in more detail for different flow rates, pulse frequencies, and mobile phase compositions. The latter was investigated to assess the influence of LC gradient elution on the spotting process. We used a four-level classification for the liquid spotting performance. Level 1 reflects accurate spotting, implying that a droplet is ejected in a straight line into the correct well according to the frequency of the solenoid valve. In level 2, droplet ejection follows the solenoid valve frequency, but a small droplet is continuously present at the end of the ejection capillary. Consequently, the direction in which the droplet is ejected is distorted and may contribute to cross-contamination. In level 3, droplet ejection does not follow the solenoid valve frequency and liquid is released in a spraylike manner. In level 4, droplets are not released, but a big drop is formed at the tip of the ejection capillary, growing in size until adhesion with the tip is overcome by gravity and it falls. A photo of solvent ejection as intended is shown in **Figure 1**. In the first step (G), the solenoid valve is closed and pressure builds up, inducing slight expansion of the elastic tubing. When the solenoid valve is opened (H), the solvent is ejected in a narrow, straight jet. Next, the valve closes (I), allowing solvent to gather again.

Table 1 shows the results obtained assessing flow rate and solvent composition according to the level classification. Especially when the water percentage is relatively

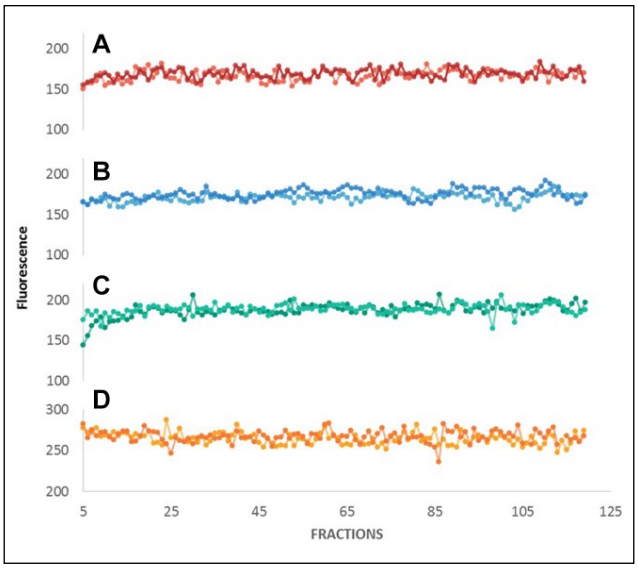

Figure 2. Fluorescence intensity of quinine per well plotted against the corresponding fraction number. Solvent water/MeOH (**A**) 98/2 or (**B**) 50/50, and water/ACN (**C**) 50/50 or (**D**) 2/98. Flow rates: (**A**) 0.1, (**B**) 0.3, (**C**) 0.6, and (**D**) 1.0 mL/min. Solenoid frequencies: (**A**) 3, (**B**) 10, and (**C,D**) 20 Hz.

high, proper droplet ejection was obtained for a large part of the flow rates tested. **Table 1** also shows that increased solenoid valve frequencies have to be applied when higher flow rates are used. Furthermore, most solvent compositions can be properly spotted with multiple frequencies. This implies, for example, that for a water/MeOH gradient at a flow rate of 0.3 mL/min, solenoid frequencies between 4 and 15 Hz will give proper results. Performing actual water/MeOH gradients for several frequencies within this range, we confirmed that these conditions allowed suitable fractionation (data not shown). Summarizing, the fractionation device is capable of addressing widely used flow rates and solvent compositions for a broad range of combinations, demonstrating its generic application in high-resolution EDA. The next steps are directed to further improving the software and enabling the user to program and adjust the solenoid frequency throughout the separation, thereby addressing situations in which the flow rate significantly changes during the analysis.

Fraction Volume Consistency

The capability of the fraction collector to consistently produce fractions of the same volume was evaluated. To this end, a solution of the fluorescent compound quinine was continuously fed into the fraction collector and fractions were deposited in a 384-well plate, of which the wells were prefilled with 100 μL of acetate buffer. After fractionation, the fluorescence intensity of each well was assessed using a plate reader. The effect of the flow rate (0.1–1.0 mL/min) and solvent composition (water/MeOH and water/ACN in different ratios) was studied at two suitable solenoid valve frequencies. As an example, **Figure 2** shows selected fluorescence readouts obtained using flow rates of 0.1, 0.3, 0.6, and 1.0 mL/min of quinine in different solvents fractionated at a specific frequency. Fractionation was carried out in duplicate. For each flow rate experiment, 115 data fractions were collected (i.e., using half a 384-well plate with the outer rows and columns left empty). As can be seen from the similar steady trend of the duplicate measurements, fraction collection is stable over time. Moreover, variation in the signal intensity among fractions is relatively small and fully random. The repeatability as expressed as the coefficient of variation (CV) for each 115-data point experiment was always lower than 3.9%. The CVs for the data shown in **Figure 2** were 3.8% (A), 3.3% (B), 3.7% (C), and 2.7% (D). Considering that these values include the variation from well plate filling with buffer and plate reader measurement, we conclude that the solenoid valve setup consistently delivers fractions of the same volume under the selected conditions.

The CV values obtained for all conditions tested (in duplicate) are summarized in **Table 2** (all plots are provided in Supplemental Information 1). For each flow rate, two frequencies were tested. The higher frequency resulted in a lower CV. Most measured CVs were equal to or below 5%, except for the lowest flow rate (0.1 mL/min) collected at low solenoid frequency (1 Hz). These conditions provided CVs ranging between 6% and 16% and were considered to be inadequate for accurate fraction collection. For 0.1 mL/min flow rates, a frequency of 3 Hz should be used, yielding CVs between 2.7% and 4.7%. In general, at lower frequencies CVs were higher. Higher frequencies result in a smaller difference in pressure between the solenoid valve closed and open positions. At low frequencies, a relatively higher pressure is built up during valve closure time, leading to more variation in deposited volume. Hence, the performance of the device generally improved when higher solenoid valve frequencies were applied. Overall, the average CV using optimal conditions was 3.7 ± 0.6. In addition to selecting a constant solenoid frequency for the collection of an LC separation, efforts are made to incorporate an option in the software that allows one to select the solenoid frequency throughout the run.

Maintenance of LC Resolution and Repeatability of Fractionation

When LC separations are fractionated, for example, for compound isolation or bioactivity screening purposes, it obviously is important that the obtained LC resolution is maintained as much as possible during fractionation.

Table 2. Fraction Collection Repeatability.

		Frequency (Hz)													
Solvent	Flow (mL/min)	1	2	3	4	5	6	7	8	9	10	15	20	30	50
Water/MeOH 98/2	0.1	15.7		3.8											
	0.3					5.0					2.8				
	0.6										3.6	3.2			
	1.0											4.3	3.2		
Water/MeOH 50/50	0.1	11.5		3.9											
	0.3					4.5					3.3				
	0.6										4.2	3.7			
	1.0											4.6	3.6		
Water/MeOH 2/98	0.1	6.0		4.7											
	0.3					4.7					4.2				
	0.6										4.7	4.5			
	1.0											4.2	4.5		
Water/ACN 98/2	0.1	8.0		2.7											
	0.3					3.9					2.7				
	0.6										3.6	3.6			
	1.0											4.1	4.1		
Water/ACN 50/50	0.1	7.7		3.7											
	0.3					4.4					3.6				
	0.6										3.7	4.9			
	1.0											5.0	4.6		
Water/ACN 2/98	0.1	9.3		4.3											
	0.3					4.0					3.3				
	0.6										3.8	3.1			
	1.0											2.5	2.7		

CV values obtained after fraction collection of a fluorescent compound dissolved in different solvent mixtures and fractionated at different flow rates and solenoid valve frequencies, in matrix format, were investigated, followed by plate reader readout.

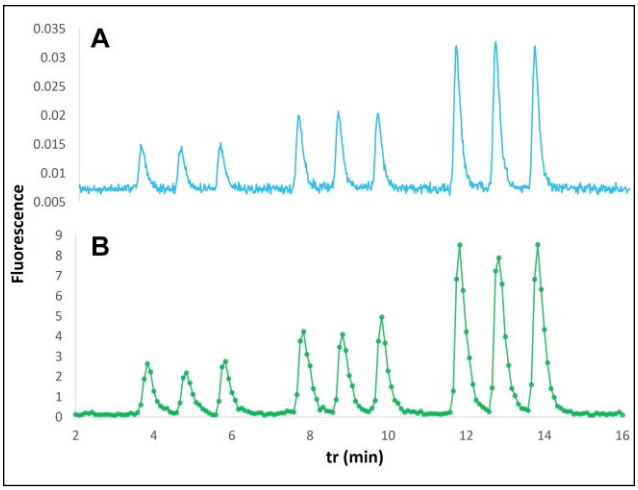

Figure 3. Successive triplicate injection of quinine solutions of 50, 100, and 150 µM, respectively. (**A**) Online fluorescence detection. (**B**) Fluorescence signal obtained by plate reader analysis after fractionation. Flow rate, 0.4 mL/min; solenoid valve frequency, 10 Hz; fraction time, 5 s.

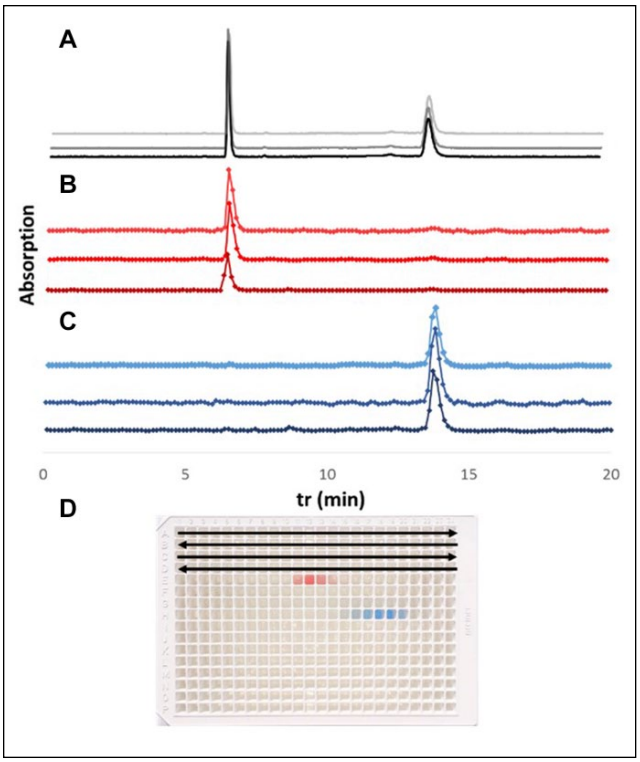

Figure 4. Triplicate LC analysis of a mixture of New Coccine and Brilliant Blue. (**A**) Chromatogram acquired by online absorbance detection. Offline chromatograms constructed from fraction analysis using a plate reader employing absorbance detection at (**B**) 500 nm and (**C**) 595 nm. (**D**) The white 384-well plate after fraction collection with the fractions comprising New Coccine (red) and Brilliant Blue (blue).

Moreover, when multiple injections are needed for analyte enrichment, it is important that the compounds of interest are consistently collected in the same fractions. To study band broadening by the fractionation process, flow injections of quinine solutions were online detected and subsequently fractionated. **Figure 3** compares the online-acquired fluorescence response of repeated injections of quinine at three different concentrations (**Fig. 3A**) with the fluorescence results obtained after fraction collection (1 fraction per 5 s) using the plate reader (**Fig. 3B**); the line widths of the plots are 1.5 points. The trace constructed from the offline measurements corresponds to the online detected trace showing no significant difference in peak shapes, widths, and intensities. This indicates that separation integrity is essentially maintained during fractionation.

To further investigate the potential band broadening and repeatability of the fractionation, a dye mixture was separated by LC and fractions of the LC effluent were collected. **Figure 4A** shows the online acquired absorbance chromatogram signal with New Coccine eluting first and Brilliant Blue second. The chromatograms constructed from the plate reader absorbance readout obtained after fractionation are depicted in **Figure 4B,C**, while the well plate after fraction collection of the dye mixture is shown in **Figure 4D**. Comparison of **Figure 4A,B** (again) shows the consistency with which the fractions were collected, reflecting the achieved LC separation and peak widths and shapes. Fractions of 3.5 s were collected, which distributed the analytes over four or five fractions with no significant band broadening. Second, the repeated fractionation of the same LC analysis of the dye mixture shows that the fractionation procedure is highly reproducible, with the analytes consistently collected in the respective fractions. This demonstrates that the fractionation device could potentially be used for analyte purification and enrichment by LC, for example, for NMR analysis of mixture components requiring significant amounts of isolated compound for successful spectral acquisition. As a proof of principle, a mixture of BPA and EE2 (100 µM each) was separated by LC (**Fig. 5A,B**) and fractionated on a 384-well plate. Only moderate fractionation resolution was used (which could also have been achieved with gravitationally based systems) to reduce dilution of the analytes over multiple fractions and allow isolation of sufficient amounts of analytes for NMR identification. Three LC separations of the mixture were fractionated on top of each other. After plate drying, the fractions that should contain the first peak as detected by UV absorbance were pooled in 700 µL of deuterated DMSO. The same was done for the peak fractions containing the second peak. Good-quality NMR spectra (**Fig. 5C,D**) could be recorded from the DMSO solutions, allowing unambiguous assignment of the first peak to BPA and the second peak to EE2. The

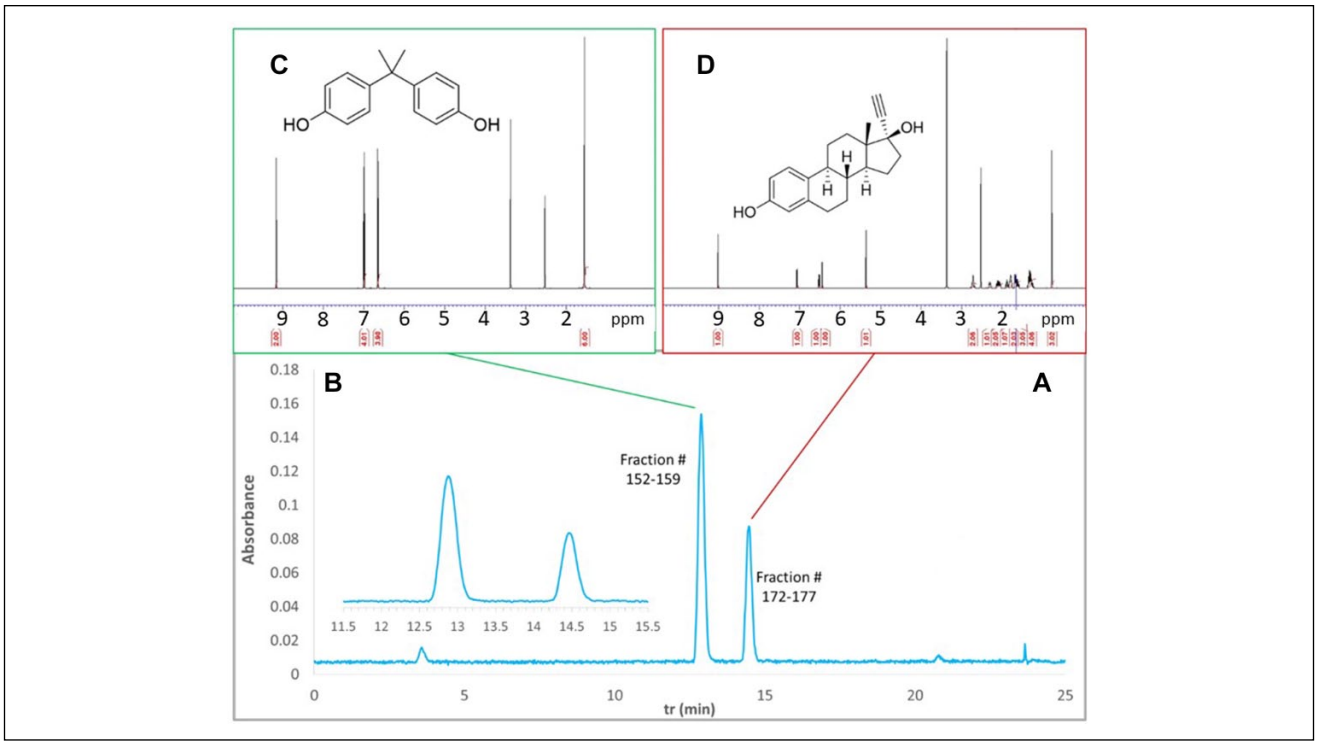

Figure 5. LC analysis and fractionation of a mixture of BPA and EE2 (100 µM each). (**A**) Chromatogram acquired by online absorbance detection with (**B**) zoom of the BPA and EE2 peaks. NMR spectra obtained for pooled fractions (**C**) 152–159 and (**D**) 172–177 after drying and reconstitution in deuterated DMSO.

isolation procedure was repeated by fractionating, each time, three LC analyses of the mixture on eight additional 384-well plates. From plates 2–4 the fractions corresponding to the first peak were pooled in 700 µL of deuterated DMSO, and so were the fractions corresponding to the second peak. In the same fashion, the respective fractions of plates 5–9 were pooled. NMR spectra of the pooled fractions were recorded. Comparing the spectra obtained by pooling analytes of one, three, and five plates, respectively, the signal-to-noise ratios increased in a linear fashion. This demonstrates that the fractionation system can be used for both isolation and enrichment of analytes permitting their NMR analysis and thus unequivocal identification.

Bioactivity Screening

To demonstrate the potential of the new fraction collector for bioactivity screening, a mixture of estrogenic and androgenic compounds was analyzed by LC and subsequently fractionated while MS detection was performed in parallel. The collected fractions were subsequently tested for estrogenic and androgenic activity with two reporter gene assays. **Figure 6A** shows the extracted-ion chromatograms obtained for the estrogenic and androgenic compounds. **Figure 6B,C** shows the bioassay chromatograms constructed from the readout of the estrogenic and androgenic assays of the collected fractions, respectively. The peaks observed in the bioassay chromatograms correlate with the peaks of the injected analytes in the extracted-ion chromatograms, indicating successful fractionation, allowing the detection of bioactives in mixtures. Interestingly, two additional bioactive peaks were observed at retention times of 12–13 min in the estrogenic activity trace (**Fig. 6B**). Correlation with the MS chromatograms revealed two small, but significant, peaks with the same *m*/*z* as E3, which may originate from related impurities. At the retention time of testosterone (an androgenic compound), a minor response in estrogenic activity was also detected (**Fig. 6B**). The structural similarity of testosterone with the endogenous estrogen estradiol may have caused some cross-reactivity. Further investigating the feasibility of using the fraction collector in environmental analysis, a surface water sample was spiked with β-E2, extracted, and analyzed by LC with fractionation bioassay detection and parallel MS detection (**Fig. 7**). The bioassay trace (**Fig. 7B**) showed a clear peak at about 14.5 min; however, in the corresponding MS chromatogram no clear mass due to β-E2 could be discerned. The most probable reason for this is that endogenous estrogenic compounds generally are difficult to ionize by ESI, and therefore the low β-E2

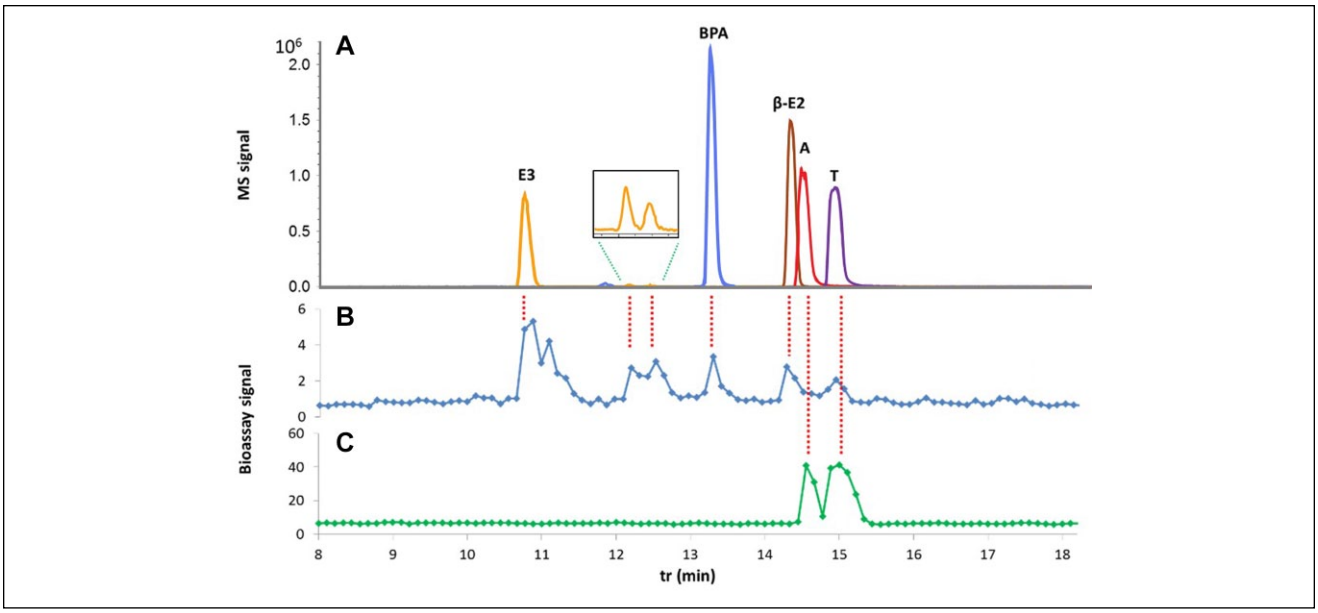

Figure 6. LC analysis of a mixture of E3, BPA, β-E2, androstenedione (A), and testosterone (T). (**A**) Extracted-ion chromatograms of the respective compounds. Constructed chromatograms from (**B**) estrogenic and (**C**) androgenic activity assay of collected fractions.

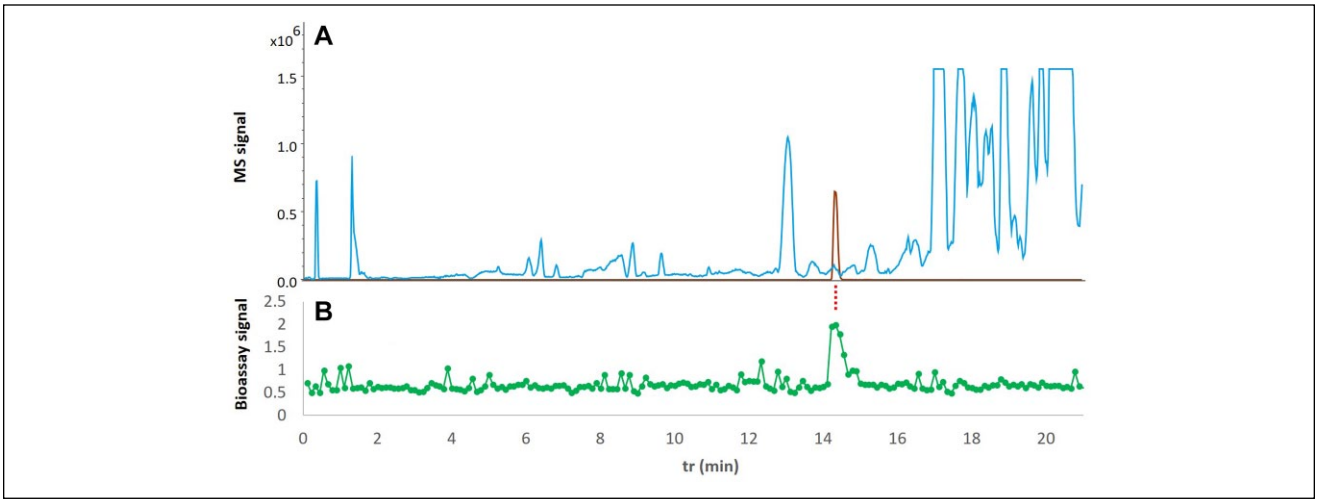

Figure 7. LC analysis of a surface water sample spiked with β-E2. (**A**) MS base-peak chromatogram (blue trace) and extracted-ion chromatogram (red trace) of β-E2. (**B**) Constructed chromatogram from estrogenic activity assay of collected fractions.

concentration could not be detected. Injection of a higher concentration of β-E2 (**Fig. 7A**, red trace) showed that the bioassay peak indeed had the same retention time as β-E2. No bioactivity peaks were found upon analysis of a blank surface water sample, while its MS chromatogram was virtually identical to that of the spiked sample.

To summarize, the development of an LC fraction collector featuring contact-free, high-frequency droplet ejection in 384-well plates using solenoid valve technology was described. The solenoid valve was implemented in an LC autosampler. The device was controlled by a dedicated software script and an electronic control unit was developed for accurate adjustment of the solenoid valve parameters. For optimization, the solenoid valve supply voltage, pulse width, and frequency in relation to the LC flow rate and solvent composition were studied. Higher LC flow rates required increased solenoid valve frequencies for optimal droplet ejection. The system's repeatability was studied by continuous supply of a fluorescent solution for fraction collection in a 384-well plate and subsequent

comparison of the fluorescence intensity of the fractions collected in buffer. The CV values obtained after plate reader analysis ranged from 2.7% to 4.7%. Fraction collection of the LC analysis of a fluorescent compound and a dye mixture showed similar widths and shapes for online detected peaks and the peaks observed in the chromatograms constructed from plate reader analysis of the deposited fractions. Furthermore, analytes were consistently collected in the same fractions without spreading to adjacent wells, showing good potential of the fractionation system for analyte purification and enrichment. The latter is of interest for offline identification of mixture components after repeated sample fractionation as demonstrated by NMR analysis of collected fractions. The new fraction collector may be of interest for the screening of environmental samples for toxicity and natural extracts for pharmacological purposes. Therefore, a mixture containing bioactive hormones and hormone-like compounds was analyzed by LC, followed by fractionation with parallel MS detection. The individual bioactives were successfully detected using two reporter gene assays, while MS detection provided their molecular mass. The device has been demonstrated to allow the accurate collection of fractions at flow rates up to 1 mL/min, while fraction widths as small as 0.5 s can be reached. Therefore, it shows good potential for fractionation of fast and high-resolution separations, as, for example, met in two-dimensional LC. The feasibility of the fraction collector for mixture component isolation followed by NMR analysis was also demonstrated.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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