## Advanced bioactivity screening analytics for rapid identification of environmental toxicants

Jonker, L.W.

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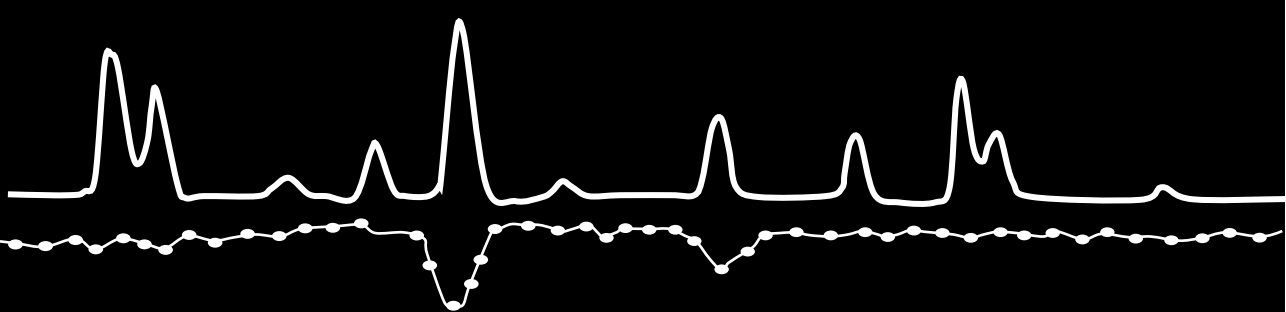

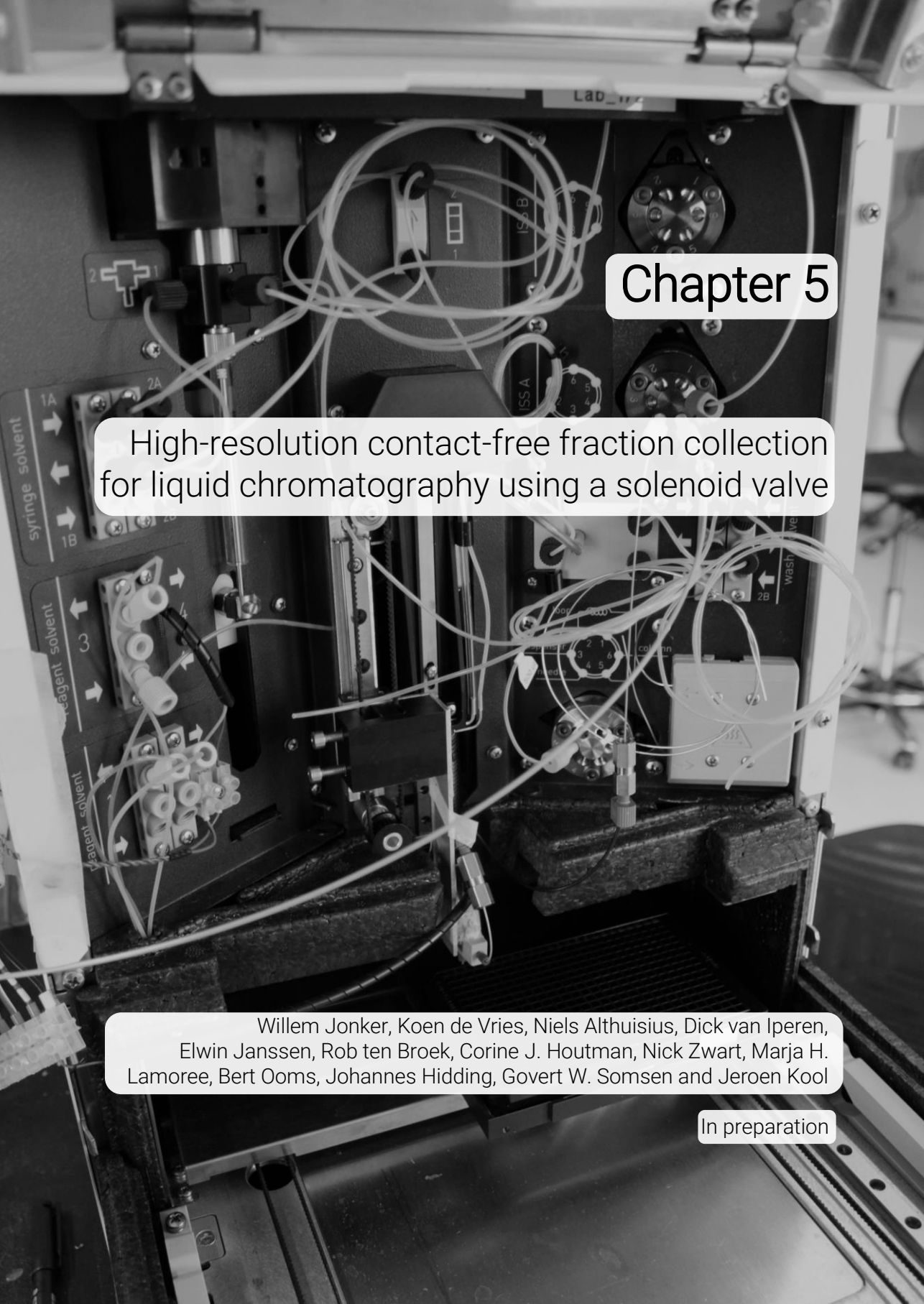


# Chapter 5

## High-resolution contact-free fraction collection for liquid chromatography using a solenoid valve

Willem Jonker, Koen de Vries, Niels Althuisius, Dick van Iperen, Elwin Janssen, Rob ten Broek, Corine J. Houtman, Nick Zwart, Marja H. Lamoree, Bert Ooms, Johannes Hidding, Govert W. Somsen and Jeroen Kool

In preparation

## Abstract

In this study we describe the development of a high resolution non-contact fraction collector for liquid chromatographic (LC) separations, allowing high-resolution fractionation in high-density well plates. The device is based on a low dead-volume solenoid valve operated at frequencies between 1 and 30 Hz for accurate collection of fractions of equal volume. The solenoid valve was implemented in a modfied autosampler and specific software and an electronic unit for valve control were developed. The influence of the solenoid supply voltage and pulse width on solvent release was determined and the effect of the fractionation frequency and flow rate and composition of the mobile phase was studied. For that 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 stable fraction collection. This was done by continuous fractionation of a fluorescent solution into a buffer solution and assessment of the fluorescence intensity of the buffer solutions after fractionation. The effluent of an LC separation of a dye mixture and a fluorescent compound was fractionated and the fractions were interrogated with UV and fluorescence detection to determine fractionation repeatability and resolution. The potential of the new fraction collector for LC-based bioactivity screening was demonstrated by fractionating the LC separation of a mixture of estrogenic and androgenic compounds, and a surface water sample (blank and spiked with bioactives). Parallel mass spectrometric detection was performed and two reporter gene assays were used 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 NMR analysis.

## 5.1 Introduction

Fraction collection of liquid chromatographic (LC) separations can be utilized for analyte purification, but also 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 [1–7], drug discovery research [8–13] and food chemistry [14–17] 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 preferably only one single 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 [18-19] for analyte identification by mass spectrometry (MS). Especially for proteomics, fraction collection of nanoLC separation on MALDI plates has been used for peptide characterization. In another approach, fractions of nanoLC separations have been collected in pipette tips and dried at room temperature, and subsequently reconstituted for chip-based direct infusion MS [20]. By disconnecting the LC separation from MS detection, different reconstitution solvents could be selected for optimal analyte ionization. Fraction collection of LC separations also finds use in analyte isolation and enrichment in many research areas for off-line analysis by nuclear magnetic resonance (NMR) spectroscopy [16-17], [21–23]. For this purpose, fractions normally are collected in vessels followed by lyophilization and reconstitution in a NMR suitable solvent [23].

Fraction collection approaches in LC vary significantly amongst 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], [16], [24]. 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 towards the collection of small-volume fractions corresponding to 2-10 s of LC time [25–27]. This approach had already shown feasible and useful in the field of drug discovery [10–12], [28].

To achieve 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-volume droplets 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 towards 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 [9]. 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 assure acceptable reproduciblity. 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. 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 on-line 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.

## 5.2 Materials and Methods

*5.2.1 Chemicals and materials*

Methanol (MeOH), acetonitrile (ACN) and formic acid (FA), all ULC-MS grade, were purchased from Biosolve B.V. (Valkenswaard, the Netherlands). Quinine hydrochloride dehydrate, acetic acid, diammonium phosphate and the colorants New coccine and Brilliant blue were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Sodium acetate trihydrate was from J.T. Baker (Deventer, The Netherlands). Well plates (white F bottom 384 well plates, white F bottom 384 well plates with transparent bottom, and black F bottom 384 well plates) were obtained from Greiner Bio-One B.V. (Alphen aan den Rijn, The 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 Thermofisher (Landsmeer, The Netherlands). Penicillin, streptomycin, fetal bovine serum (FBS) charcoal stripped (FBS), dimethyl sulfoxide, D-Luciferin, tris(hydroxymethyl)aminomethane (TRIS), dithiotreitol (DTT), glycerol, Triton-X100, 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 (Zwijndrecht, The Netherlands).

*5.2.2 Fraction collector*

The solenoid-valve based fraction collector was constructed from a Spark Holland Integrity autosampler (Emmen, The Netherlands) in which a Bürkert type 6712-2/2 way whisper valve (Breda, The Netherlands) was mounted for high frequency droplet generation. Figure 1 shows the device highlighting the location of the solenoid valve (A) and the low-dead-volume channel connection piece made from Perspex. The latter unit allows connection of the solenoid valve with 1/32" LC tubing (inlet) and a deactivated fused-silica capillary of 3 cm length and 250 µm i.d. (outlet). Two white O-rings ensure leak-tight connection between the solenoid valve and the connection piece. The final version of the connection piece was made of PEEK providing chemical resistance against organic solvents and material robustness. The solenoid valve was mounted on a stainless steel unit that also guides the electric wires and solvent tubing towards the solenoid valve. The stainless steel mounting unit was connected to an assembly (B) that is gripping on one side of the cord (C) which can be moved vertically to allow adjustment of the space between the solenoid valve and the well plate (D). The switch valve (E) is used to direct the LC flow either to the waste or the solenoid

valve. An external electronic signal converter (F) was developed for accurate control of the solenoid valve in terms of frequency and pulse width supplied the solenoid valve with an increased voltage (up to 28 V) for solvent ejection. The modifications to the device have been performed such that the original autosampler function was maintained.

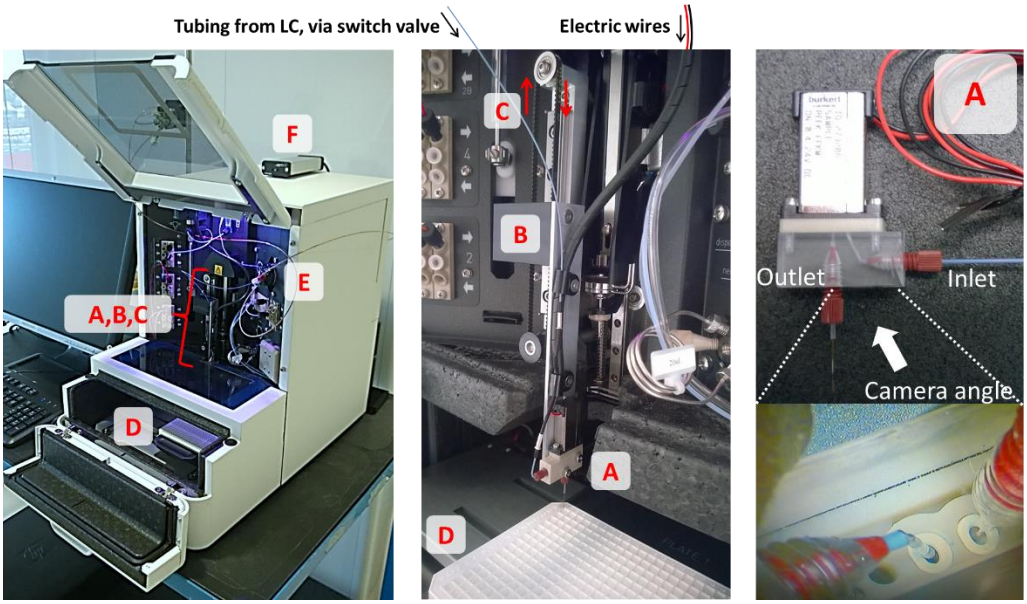


*Figure 1. Solenoid-valve based fraction collector. (A) Solenoid valve with connection piece enabling connection with LC tubing (inlet) and a deactivated fused silica capillary (outlet). (B) Assembly connecting the stainless steel mounting piece for the solenoid valve with the string for height adjustment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

*5.2.3 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. In order 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 2-11 ms in steps of 1 ms.

*5.2.4 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 applied 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).

*5.2.5 Fraction collector 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 0.1 M sodium acetate buffer (pH 5) per well. For each experiment 120 fractions were collected. Subsequently, the fluorescence signal ($\lambda_{ex}$ 330 nm, $\lambda_{em}$ 380 nm) of each fraction was measured with a Thermo VarioSkan plate reader (Breda, The Netherlands). Six different combinations of commonly applied 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 filled with the test solution was connected to a Shimadzu LC20AD pump (Breda, The Netherlands) operated at 0.1, 0.3, 0.6 or 1.0 ml/min.

*5.2.6 Sample fractionation and detection, resolution and repeatability assessment*

To study fraction collection resolution, the LC analysis of quinine was fractionated (50, 100 and 150 µM) was fractionated in triplicate using a Waters XBridge BEH C18 coumn, (1.5 µm; 4.6. x 100 mm (Etten-Leur, The 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, and a Shimadzu RF-10axl fluorescence detector (Breda, The Netherlands). Fractions were collected in 384 well plates filled with 100 µl 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 $\lambda_{ex}$ 330 nm and $\lambda_{em}$ 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, 2.1x50 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 (pH8) [29]. The gradient started at 10% B which was held constant for 3 min, subsequently increased to 95% within 4 min and maintained at this composition for another 5 min. Next, the %B was returned to 10% and held constant for 5 min. The column effluent was directed via a

Shimadzu SPD20AV UV/VIS detector (Breda, The Netherlands) 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.

*5.2.7 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) was extracted in duplicate following a previously published procedure [26]. The concentration β-E2 in the spiked-water extract was 2.6 nM (after extraction). LC analysis was performed on a Thermo Scientific Dionex UltiMate 3000 ultrahigh performance liquid chromatography system (Amsterdam, The Netherlands) equipped with a Waters ACQUITY UPLC BEH C18 Column (130Å, 1.7 µm, 2.1x150 mm). Milli-Q water and MeOH were used for LC separation at a flow of 0.4 ml/min and 55 $^{0}$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 towards a Bruker Impact II time-of-flight mass spectrometer with an electrospray ionization (ESI) source and 90% towards the developed 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; dry gas pressure, flow and temperature, 2 bar, 8.0 l/min and 200 $^{0}$C, respectively, for both polarities. The mass analyzer settings for detection in positive mode were: both funnels at 150 peak to peak voltage (Vpp); hexapole 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; prepulse storage, 5 µs. Negative mode: both funnels at 200 peak to peak voltage (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; pre-pulse storage, 5 µs. Fractions of 6.5 s were collected in 384-well plates. The protocol of Rogers and Denison was followed for cell culturing of the VM7Luc4E2 cells [30]. The AR-Ecoscreen cells were cultured as described by Araki et al. [31]. 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 [26] with modifications (in 384-well plate format). In brief, cells were seeded at a

concentration of 200,000 cells/ml in white F 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 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 volume of 50 µl 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 pinpointing of bioactive peaks.

*5.2.8 Fraction collection for NMR analysis*

A mixture of EE2 and BPA (200 µM each; 50 µl injected) was analysed on a Waters XBridge BEH C18, column (3.5 um, 2.1x50 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. The mixture was analysed and fractionated in triplicate using one well plate with overlayed fractions. This procedure was repeated yielding a total of 27 analyses fractionated on 9 well plates (each plate containing 3 analyses). Plates were dried with a vacuum centrifuge. Fractions from plate 1 containing BPA were reconstituted and gathered in 700 µl deuterated DMSO. The same was done for the the fractions containing EE2. The fractions containing EE2 or BPA from plates 2, 3 and 4 were pooled in a final volume of 700 µl. The same was performed for plates 5, 6, 7, 8 and 9. From the obtained samples NMR spectra were recorded on a Bruker Avance 500 using the residual solvent peak as 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), 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, $^1$J = 8.40 Hz, $^2$J = 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, $^1$J = 12.72 Hz, $^2$J = 3.47 Hz), 1.82-1.73 (m, 2H), 1.71-1.56 (m, 3H), 1.37-1.20 (m, 4H), 0.76 (s, 3H).

## 5.3 Results and Discussion

A solenoid valve was implemented in an autosampler device in order 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 function of mobile phase flow rates and compositions.

### *5.3.1 Solenoid valve parameters*

Different solenoid-valve pulse widths were tested to assess the influence on droplet release. A pulse width of 4 ms showed optimal for solenoid-valve frequencies 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 small 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 aided droplet ejection.

### *5.3.2 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 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 is not following the solenoid valve frequency and liquid is released in a spray like 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 image of solvent ejection as intended is given in Figure 2. In the first step, the solenoid valve is closed and a pressure builds up inducing slight expansion of the elastic tubing. When the

solenoid valve is opened (step 2), the solvent is ejected in a narrow, straight jet. Next, the valve closes (step 3) allowing solvent to gather again.

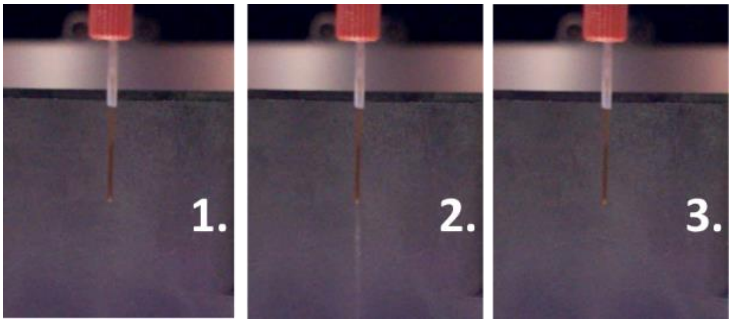


*Figure 2. Solvent ejection (1) Solenoid valve closed; (2) Solenoid valve open, ejection of solvent in a narrow beam; (3) Solenoid valve closed.*

Table 1 shows the results obtained assessing flow rate and solvent composition according the level classification. Based on visual inspection, clearly, there are multiple frequencies at which the solenoid valve properly released droplets. Especially when the water percentage is relatively high, proper droplet ejection was obtained for 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, confirmed these conditions allowed suitable fractionation (data not shown).

*Table 1. Assessment of droplet ejection using classification by 4 levels. (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 result of continuous presence of 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 eventual release due to gravitational force.*

| Solvent. | Freq (Hz) → 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 |

*5.3.3 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 acetate buffer. After fractionation, the fluorescence intensity of each well was assessed using a plate reader. Effect of 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 3 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 4 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 set-up consistently delivers fractions of the same volume under the selected conditions.

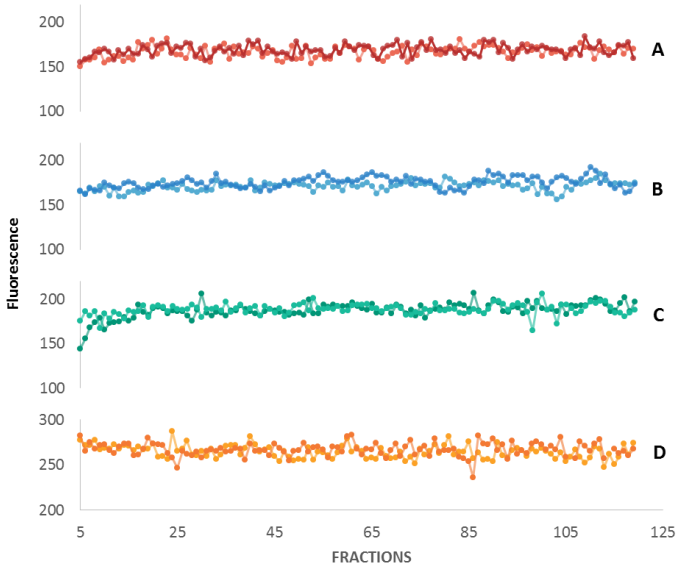


*Figure 3.*
*Fluorescence intensity of quinine per well plotted against the corresponding fraction number. Solvent water/MeOH 98/2 (A) or 50/50 (B), and water/ACN 50/50 (C) or 2/98 (D); Flow rate, 0.1 (A), 0.3 (B), 0.6 (C) and 1.0 (D) ml/min; Solenoid frequency, 3 (A), 10 (B) and 20 (C+D) Hz.*

The CV values obtained for all conditions tested (in duplicate) are summarized in Table 2 (all plots are provided in SI1). For each flow rate, two frequencies were tested. The higher frequency predominantly resulted in a lower CV. Most measured CVs were equal 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 preferably be used, yielding CVs between 2.7% and 4.7%. At lower frequencies CVs generally were higher. Higher frequencies, result in a smaller difference in pressure between the solenoid valve closed and open positions. At low frequencies, a relatively high 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.

*Table 2. Fraction collection repeatability. 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 investigated, followed by plate reader readout.*

| Solvent | Freq (Hz) → 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 | | |

*5.3.4 Maintenance of LC resolution and repeatability of fractionation*

When LC separations are fractionated for e.g. compound isolation or bioactivity screening purposes, it obviously is important that the obtained LC resolution is maintained as much as possible during fractionation. Moreover, when multiple injections are needed for analyte enrichment, it is important that the compound(s) of interest are consistently collected in the same fractions. In order to study band broadening by the fractionation process, flow injections of quinine solutions were online detected and subsequently fractionated. Figure 4 compares the online-acquired fluorescence response of repeated injections of quinine at three different concentrations (Figure 4A) with the fluorescence results obtained after fraction collection (1 fraction per 5 s) using the plate reader (Figure 4B). The trace constructed from the offline measurements nicely resembles the online detected trace showing no significant difference in peak shapes, widths and intensities. This indicates that separation integrity is essentially maintained during fractionation.

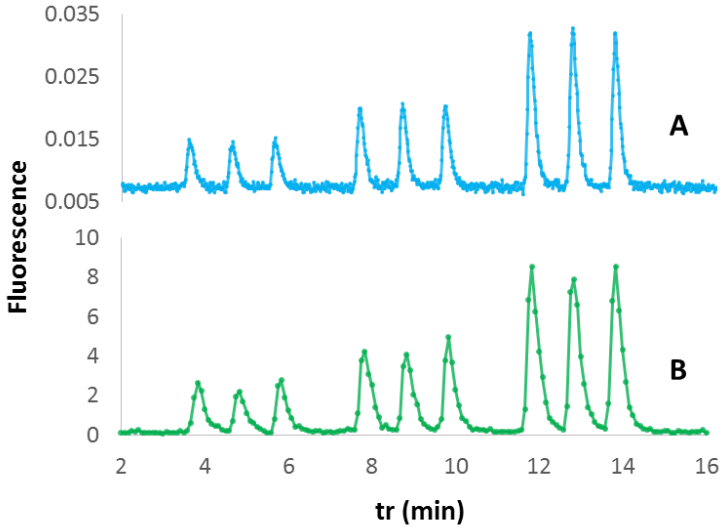


*Figure 4. Successive triplicate injection of quinine solutions of, respectively, 50, 100 and 150 µM. (A) on-line fluorescence detection. (B) Fluorescence signal obtained by plate reader analysis after fractionation. Flow rate, 0.4 ml/min; solenoid valve frequency, 10 Hz, fraction time, 5 s.*

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 5A shows the online acquired absorbance chromatograms 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 Figures 5B and 5C, while the well plate after fraction collection of the dye mixture is shown in Figure 5D. Comparison of Figures 5A-C first, (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 4-5 fractions with no significant band broadening. Secondly, 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 e.g. 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 (Figures 6A and 6B) and fractionated on a 384-well plate. In order to allow isolation of sufficient amounts of analytes for NMR identification, three LC analyses 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 deuterated DMSO. The same was done for the peak fractions containing the second peak. Good-quality NMR spectra (Figures 6C and 6D) could be recorded from the DMSO solutions, allowing unambiguous assignment of the first peak to BPA and the second peak to EE2. The isolation procedure was repeated by fractionating each time three LC analyses of the mixture on eight additional 384-well plates. From plates 2, 3 and 4 the fractions corresponding to the first peak were pooled in 700 µl deuterated DMSO, and so were the fractions corresponding to the second peak. In the same fashion, the respective fractions of plates 5, 6, 7, 8, and 9 were pooled. NMR spectra of the pooled fractions were recorded. Comparing the spectra obtained by pooling analytes of respectively 1, 3 and 5 plates, 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.

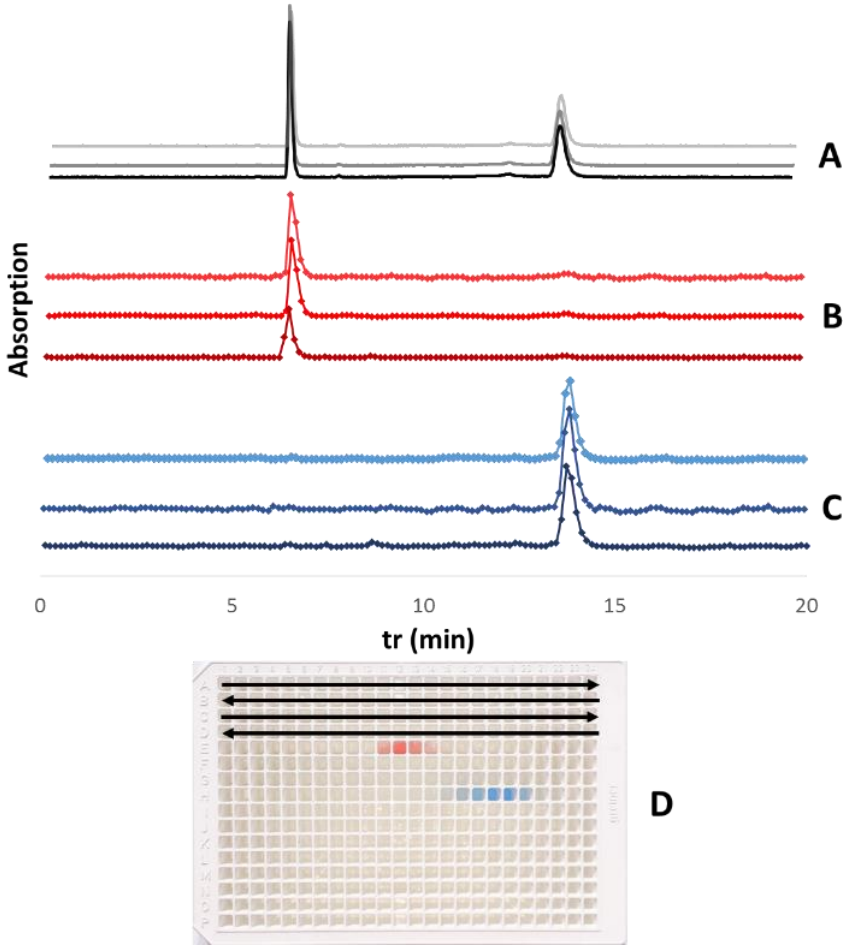


*Figure 5. Triplicate LC analysis of a mixture of New coccine and Brilliant blue. (A) Chromatogram acquired by online absorbance detection; (B+C) Offline chromatograms constructed from fraction analysis using a plate reader employing absorbance detection at (B) 500 nm and (C) 595 nm; (D) The white 384-wells plate after fraction collection with the fractions comprising New coccine (in red) and Brilliant blue (in blue).*

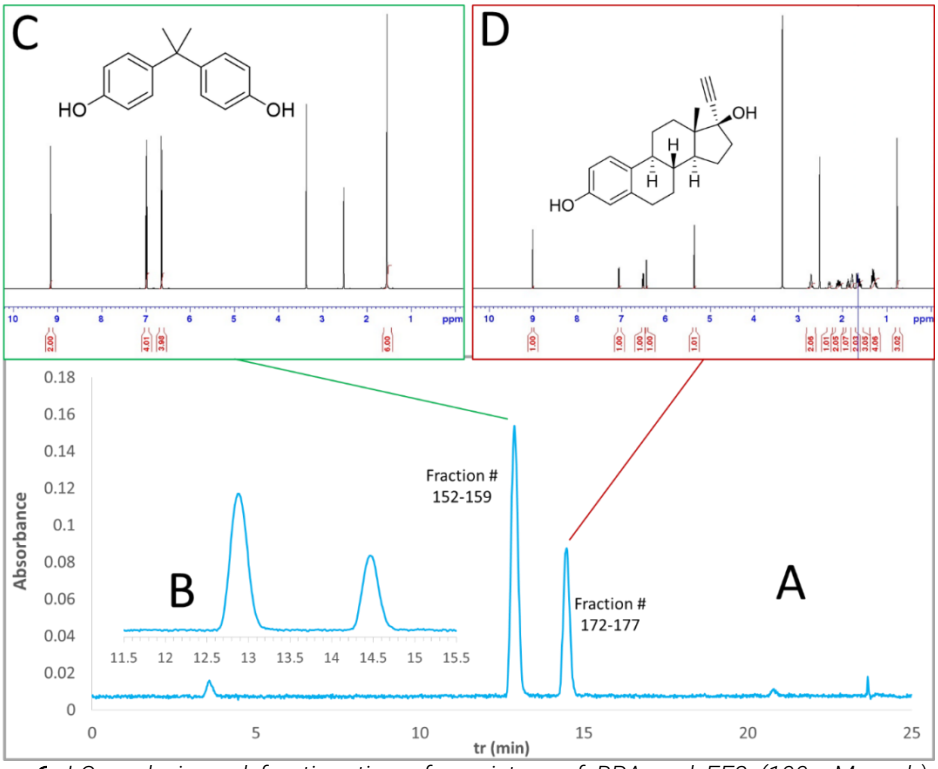


*Figure 6. LC analysis and fractionation of a mixture of BPA and EE2 (100 uM each). (A) Chromatogram acquired by online absorbance detection with (B) zoom of the BPA and EE2 peaks; (C+D) NMR spectra obtained for pooled fractions 152-159 (C) and 172-177 (D) after drying and reconstitution in deuterated DMSO.*

### *5.3.5 Bioactivity screening*

In order to demonstrate the potential of the new fraction collector for bioactivity screening, a mixture of estrogenic and androgenic compounds was analysed 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 7A shows the extracted-ion chromatograms obtained for the estrogenic and androgenic compounds. Figures 7B and 7C show 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 nicely correlate with the peaks of the injected analytes in the extracted-ion chromatograms, indicating successful fractionation allowing detection of bioactives in mixtures. Interestingly, two additional bioactive peaks were observed at retention times of 12-13 min in the estrogenic activity trace (Figure 7B). Correlation with the MS chromatograms revealed two small, but significant, peaks with the same m/z as estriol, which may originate from related impurities.

At the retention time of testosterone (an androgenic compound), also a minor response in estrogenic activity was detected (Figure 7B). 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-estradiol, extracted and analysed by LC with fractionation-bioassay detection and parallel MS detection (Figure 8). The bioassay trace showed a clear peak at ca. 14.5 min, however, in the corresponding MS chromatogram no clear mass due to β-estradiol could be discerned. Most probable reason for this is that endogenous estrogenic compounds generally are difficult to ionize by ESI and therefore the low β-estradiol concentration could not be detected. Injection of a higher concentration of β-estradiol (Figure 8A, red trace) showed that the bioassay peak indeed had the same retention time as β-estradiol. 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.

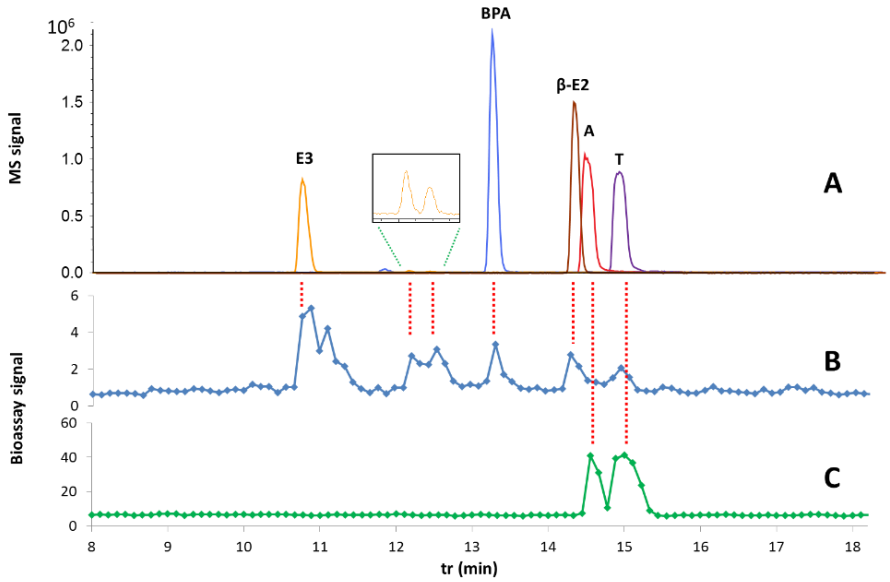


*Figure 7. LC analysis of a mixture of estriol (E3), bisphenol A (BPA), β-estradiol (β-E2), androstenedione (A) and testosterone (T). (A) extracted-ion chromatograms of the respective compounds; (B+C) constructed chromatograms from estrogenic (B) and androgenic (C) activity assay of collected fractions.*

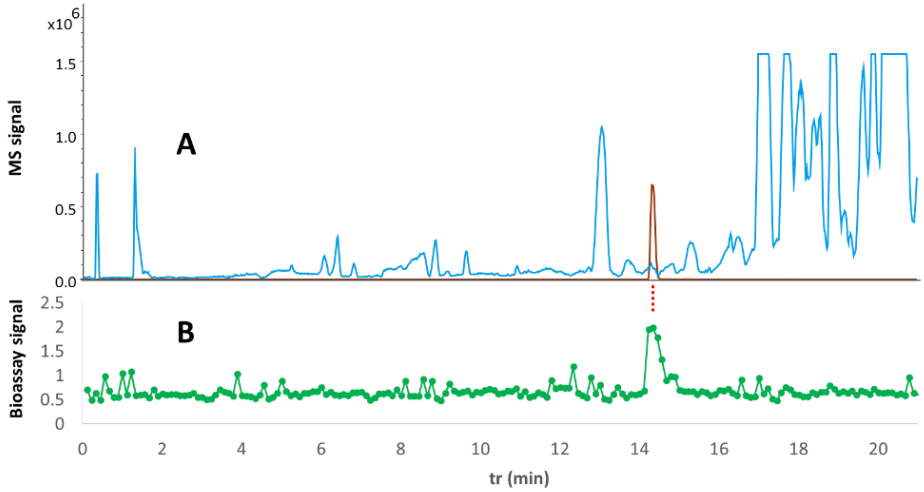


*Figure 8. LC analysis of a surface water sample spiked with β-estradiol. (A) MS base-peak chromatogram (blue trace) and extracted-ion chromatogram (red trace) of β-estradiol; (B) constructed chromatogram from estrogenic activity assay of collected fractions.*

## 5.4 Conclusions

The development of an LC fraction collector featuring contact-free high-frequency droplet ejection in 384 well plates using solenoid-valve technology is 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% and 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 screening and natural extracts for pharmacological purposes.

Therefore, a mixture containing bioactive hormones and hormone-like compounds was analysed 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 demonstrated to allow accurate collection of fractions at flow rates up to 1 ml/min, while fractions as low as 0.5 s can be reached. Therefore, it shows good potential for fractionation of fast and high-resolution separations, as e.g. met in two-dimensional LC. The feasibility of the fraction collector for mixture component isolation followed by NMR analysis was also demonstrated.

Acknowledgements
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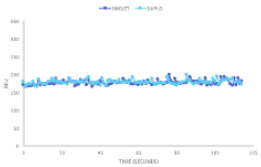
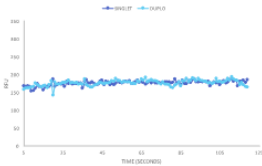
**Supplementary information** ***S-1.*** *Fraction volume consistency.*

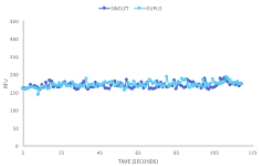
Flow: 0,6 ml/min
Freq.: 10 Hz Int.: 1 sec



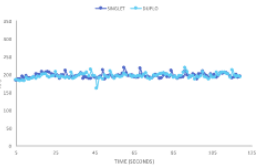
| | SINGLET | DUPLO |
|---|---|---|
| Average | 176,8 | 178,6 |
| SD | 6,6 | 6,1 |
| RSD | 3,7% | 3,4% |



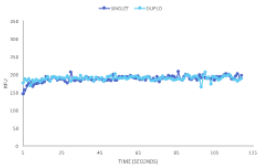
| | SINGLET | DUPLO |
|---|---|---|
| Average | 175,2 | 175,8 |
| SD | 8,6 | 7,9 |
| RSD | 4,8% | 4,5% |



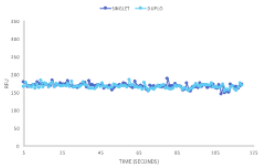
| | SINGLET | DUPLO |
|---|---|---|
| Average | 169,7 | 171,5 |
| SD | 7,9 | 7,8 |
| RSD | 4,7% | 4,8% |



| | SINGLET | DUPLO |
|---|---|---|
| Average | 196,4 | 195,5 |
| SD | 6,5 | 7,5 |
| RSD | 3,3% | 3,8% |

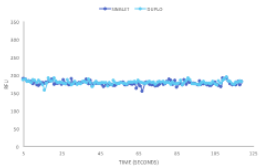


| | SINGLET | DUPLO |
|---|---|---|
| Average | 187,5 | 188,3 |
| SD | 7,7 | 6,0 |
| RSD | 4,1% | 3,2% |

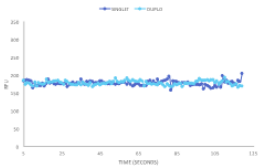


| | SINGLET | DUPLO |
|---|---|---|
| Average | 167,2 | 166,1 |
| SD | 6,7 | 6,1 |
| RSD | 4,0% | 3,6% |

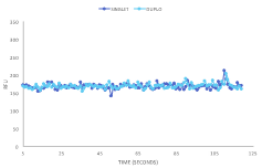
Flow: 0,6 ml/min
Freq.: 20 Hz Int.: 1 sec



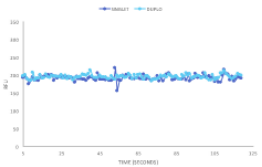
| | SINGLET | DUPLO |
|---|---|---|
| Average | 176,2 | 178,6 |
| SD | 6,0 | 5,3 |
| RSD | 3,4% | 2,9% |



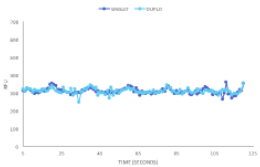
| | SINGLET | DUPLO |
|---|---|---|
| Average | 176,7 | 177,3 |
| SD | 7,0 | 5,9 |
| RSD | 4,0% | 3,3% |



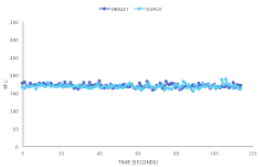
| | SINGLET | DUPLO |
|---|---|---|
| Average | 168,0 | 168,8 |
| SD | 8,1 | 7,1 |
| RSD | 4,8% | 4,2% |



| | SINGLET | DUPLO |
|---|---|---|
| Average | 191,8 | 195,5 |
| SD | 7,8 | 5,8 |
| RSD | 4,1% | 3,0% |

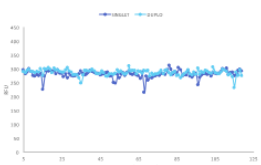


| | SINGLET | DUPLO |
|---|---|---|
| Average | 308,5 | 308,8 |
| SD | 15,7 | 14,3 |
| RSD | 5,1% | 4,6% |



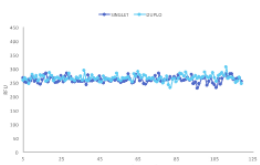
| | SINGLET | DUPLO |
|---|---|---|
| Average | 169,5 | 167,6 |
| SD | 5,1 | 5,3 |
| RSD | 3,0% | 3,2% |

Flow: 1,0 ml/min
Freq.: 15 Hz Int.: 1 sec

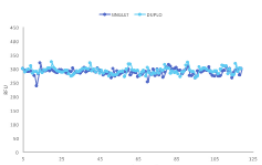


| | SINGLET | DUPLO |
|---|---|---|
| Average | 279,4 | 285,2 |
| SD | 13,5 | 10,5 |
| RSD | 4,8% | 3,7% |

| | SINGLET | DUPLO |
|---|---|---|
| Average | 280,3 | 291,4 |
| SD | 14,3 | 11,6 |
| RSD | 5,1% | 4,0% |

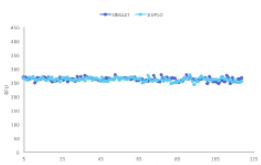


| | SINGLET | DUPLO |
|---|---|---|
| Average | 258,5 | 265,8 |
| SD | 10,9 | 11,0 |
| RSD | 4,2% | 4,2% |



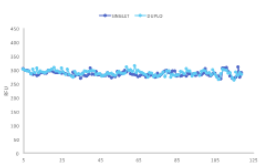
| | SINGLET | DUPLO |
|---|---|---|
| Average | 287,3 | 291,5 |
| SD | 11,6 | 11,6 |
| RSD | 4,1% | 4,0% |

| | SINGLET | DUPLO |
|---|---|---|
| Average | 264,7 | 271,3 |
| SD | 15,2 | 11,7 |
| RSD | 5,7% | 4,3% |

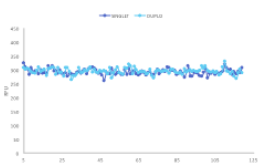


| | SINGLET | DUPLO |
|---|---|---|
| Average | 260,8 | 259,6 |
| SD | 6,7 | 6,2 |
| RSD | 2,6% | 2,4% |

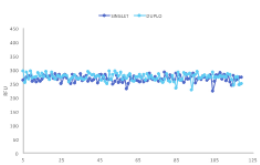
Flow: 1,0 ml/min
Freq.: 20 Hz Int.: 1 sec



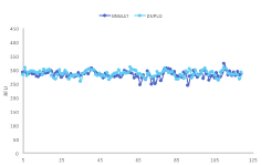
| | SINGLET | DUPLO |
|---|---|---|
| Average | 284,6 | 286,4 |
| SD | 8,9 | 9,3 |
| RSD | 3,1% | 3,2% |



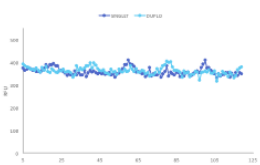
| | SINGLET | DUPLO |
|---|---|---|
| Average | 291,8 | 294,7 |
| SD | 10,3 | 10,9 |
| RSD | 3,5% | 3,7% |



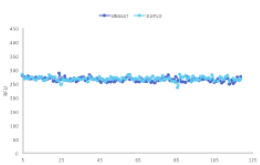
| | SINGLET | DUPLO |
|---|---|---|
| Average | 268,0 | 273,2 |
| SD | 11,8 | 12,6 |
| RSD | 4,4% | 4,6% |



| | SINGLET | DUPLO |
|---|---|---|
| Average | 282,7 | 286,4 |
| SD | 12,2 | 11,2 |
| RSD | 4,3% | 3,9% |



| | SINGLET | DUPLO |
|---|---|---|
| Average | 317,2 | 361,2 |
| SD | 16,7 | 15,8 |
| RSD | 4,7% | 4,4% |



| | SINGLET | DUPLO |
|---|---|---|
| Average | 264,1 | 266,8 |
| SD | 7,2 | 7,3 |
| RSD | 2,7% | 2,7% |

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