Chapter V

Microfluidic micromixer as a tool to overcome solvent incompatibilities in two-dimensional liquid chromatography

Margaryta A. Ianovska1,2, Arto Heiskanen3, Bert Wouters4, Erik Ritzen5, Ynze Mengerink5, Peter Schoenmakers4, Jenny Emnéus3, Elisabeth Verpoorte1

1Pharmaceutical Analysis, Groningen Research Institute of Pharmacy, University of Groningen, The Netherlands
2TI-COAST, Amsterdam, The Netherlands
3Department Micro- and Nanotechnology, Technical University of Denmark, Denmark
4University of Amsterdam, Van ‘t Hoff Institute for Molecular Sciences, Amsterdam, The Netherlands
5DSM Resolve, Geleen, The Netherlands

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Abstract

We report a micromilled, pressure-resistant mixing chip for application in on-line comprehensive two-dimensional liquid chromatography (LC×LC). This microfluidic mixer is based on chaotic advection generated in grooved microchannels, and can be operated at flow rates compatible with LC×LC (0.1-1 mL/min). The design of this chip was optimized and tested in our previous work in the transparent and flexible silicon rubber material, polydimethylsiloxane (PDMS). In this work, the microfluidic chip was micromilled in rigid cyclic-olefin copolymer (COC) substrate in order to better withstand the high pressure environment of LC×LC. Two micromilled parts were bonded using solvent-vapour-assisted bonding under elevated temperature and pressure. A specially designed, robust, low-dead-volume interface allows direct connection of the microfluidic chip to an LC×LC system using standardized HPLC connectors. Thus-fabricated chips can withstand pressures of 200 bar. The chip was successfully implemented in a comprehensive two-dimensional HILIC×RP-LC system for improved separation and identification of various oligomeric series in nylon polymer (polyamide) samples. Initial trials of the micromixer in a simple modulator at the interface between the two columns yielded chromatographic performance similar to that obtained with commercially available mixing units. However, with the grooved micromixer, it was possible to mix rapidly within the same flow rate range in a volume, which was 30 times smaller than the commercially available micromixers, opening a route to utilization of microfluidic mixers in the conventional equipment.

Keywords: microfluidic chip-based technologies; cyclic-olefin copolymer (COC) devices; micromilling; two-dimensional liquid chromatography (LC×LC).
Introduction

With the growing complexity of real samples, there is a need to analyze these in the most complete way possible. While liquid chromatography is a powerful separation tool, a single column does not offer sufficient resolution for such samples, which can, for example, contain many different proteins, peptides or inorganic polymers. This has led to the rapid development of techniques that employ combinations of columns with different separation mechanisms and thus different selectivity, such as two-dimensional liquid chromatography (2D-LC).\(^1\,^2\) In order to maintain the resolution obtained in the first dimension (\(1\text{D}\)) in the second dimension (\(2\text{D}\)), the two dimensions are coupled through a special interface that performs all the required manipulations of the \(1\text{D}\) effluent, such as sampling, storing and re-injection of small \(1\text{D}\) effluent fractions. In comprehensive two-dimensional (2D) liquid chromatography (LC×LC), where all sample material passes through both columns before reaching the detector,\(^3\) it is especially important that the switching frequency of the interface is high. The smaller the volumetric sample fractions are and the faster they are transferred to the \(2\text{D}\), the better the \(1\text{D}\) resolution is maintained.

Each separation mechanism in LC×LC requires its own mobile phase for the best separation of analytes. The utilization of different stationary phases and, as a consequence, different mobile phases in LC×LC means that the fractions dissolved in the \(1\text{D}\) mobile phase need to be transferred to the second dimension with a mobile phase having a different composition. Ideally, the second-dimension (\(2\text{D}\)) mobile phase should be a stronger eluting mobile phase on the \(2\text{D}\) column than the \(1\text{D}\) mobile phase at the time of elution. In addition, the \(2\text{D}\) column should have a higher retention with this latter mobile phase than the \(1\text{D}\) column. Also, the \(1\text{D}\) effluent and the \(2\text{D}\) mobile phase at the moment of injection should be fully miscible. If these conditions are fulfilled the band broadening caused by mobile phase incompatibility is minimized.\(^4\) However, such a combination of mobile and stationary phases is usually difficult to achieve, especially in highly orthogonal systems, such as hydrophilic-interaction liquid chromatography (\(1\text{D}\)) followed by reversed-phase liquid chromatography (\(2\text{D}\)) (HILIC×RP-LC). In this case, the \(1\text{D}\) effluent is rich in organic solvent, giving it a high elution strength in the RP dimension. If the order of columns were to be reversed, the water-rich effluent from the \(1\text{D}\) RP column would act as a strong eluent in a \(2\text{D}\) HILIC system. Hence, the solvent in the collected \(1\text{D}\) fraction is a stronger eluent on the \(2\text{D}\) column than the \(2\text{D}\) mobile
phase,\textsuperscript{5} which can cause a decrease in retention, increased band broadening, and non-symmetrical or even split peaks.\textsuperscript{6,7}

To overcome the solvent-strength mismatch problem between the dimensions in LC×LC, a number of solutions have been previously suggested, including trapping-column interfaces\textsuperscript{8–10} and collection of very small fraction volumes from a 1D capillary monolithic column.\textsuperscript{7} More sophisticated approaches utilize a vacuum-evaporation interface for on-line evaporation of 1D solvent from the loop\textsuperscript{11} or thermally assisted modulation using the influence of temperature on the retention of analytes.\textsuperscript{12,13} While each of these approaches has unique advantages for solving the problem of mobile-phase incompatibility, these technologies are still not developed to the extent where they could be used universally in routine LC×LC analyses.

An approach which is more generally applied is the use of an additional solvent flow (make-up flow) to allow modification of the solvent composition between dimensions by diluting the collected 1D fraction with a weaker 2D mobile phase. However, this requires the use of a good mixing device at the interface between the two columns. Moreover, the mixer must be able to withstand brief pressure pulses up to a few hundred bar, due to its connection to switching valves used to shuttle sample from the 1D to the 2D. Nowadays, T-pieces\textsuperscript{14} and static mixers (S-mixers)\textsuperscript{15,16} are used for this purpose. A T-piece mixer provides mixing by causing two streams to collide at a T-junction to create an interdiffusion region where mixing by diffusion takes place more rapidly.\textsuperscript{17} S-mixers, on the other hand, are based on two elements in a circular tube, each consisting of multiple X-shaped cross-bars positioned at 45° with respect to the axis of the tube, but with the second element rotated 90° with respect to the first.\textsuperscript{18} These two elements form a unit which is repeated a number of times. Mixing is achieved by splitting the main flow into sub-streams and then re-joining them. Due to this split-and-recombine mechanism, the diffusion length between the two fluids decreases due to an increase in the contact surface area. Both T-piece and S-mixer approaches exhibit better performance at higher flow rates and larger mixing-unit volumes.\textsuperscript{19} For the flow-rate range (100-300 µL/min) that is used for most 2D-LC separations, the volume of the suitable mixer should be at least 150 µL (for S-mixer). Such volumes are not in keeping with the low 1D fraction volumes that have to be manipulated, which makes the application of these mixing units in LC×LC not efficient enough. 2D-LC thus stands to benefit from the development of fast, small-volume micromixers.

The development of chip-based devices integrated in LC or LC-MS systems using microfluidic technology has increased substantially in the last decade.\textsuperscript{20–25} The key advantages of chip-based microfluidic systems are their small size, resulting in small-volume devices, and
their applicability for controlling small amounts of liquids, which is beneficial for application in LC. However, many chip-based devices implemented in LC and LC-MS systems are operated in nano-LC mode at flow rates in the 100-300 nL/min range, whereas the conventional LC×LC applications imply utilization of higher flow rates. The connection to conventional equipment operating at high pressures implies that devices should be also pressure-resistant. In previous work, we developed a 1.6-μL mixer with herringbone grooves (also referred to grooves having an asymmetric chevron geometry) that provides good mixing within seconds at flow rates compatible with LC×LC (0.1-1 mL/min). This mixer has a small internal volume in order to obtain the desired dilution ratios in minimal volumes. The mixing is achieved through the generation of two counter-rotating vortices (perpendicular to the direction of the main flow) based on chaotic advection, a substantially different mechanism than in T-piece and S-mixers. However, in that research we employed poly(dimethylsiloxane) (PDMS), a transparent and flexible silicone rubber, the elastomeric nature and low Young's modulus of which become a significant problem for development of high-pressure-resistant chips. At high flow rates, significant channel deformations (resulting from high-pressure flows) eventually lead to the fatigue and cracking of PDMS.

In order to cope with the pressures used in conventional LC systems, we fabricated our micromixer with herringbone grooves in cyclic-olefin copolymer (COC) using micromilling. We decided to use COC, because of its compatibility with typical solvents used in HPLC (e.g. acetonitrile, methanol, and isopropyl alcohol) and excellent optical and mechanical properties. Moreover, COC has substantially lower raw material costs (compared to silicon and glass) and can be easily used in conjunction with micromilling. This fabrication method was chosen due to its applicability for the rapid translation of designs into prototypes, and it met our requirements with respect to cost and resolution as well.

Beside the need for the chip itself to withstand pressure for the LC applications, a connection between conventional equipment and the chip, the so-called macro-to-micro interface, should be pressure-resistant. However, there are only a few articles that propose pressure-resistant interfaces compatible with LC equipment. Wouters et al. developed a macro-to-micro interface for connection of a COC chip to LC equipment using a custom-made aluminium chip holder with integrated flat-bottom Nanoport connections to connect 360-μm-o.d. capillary PEEK tubing to the chip. The burst pressure (the pressure that a device can withstand before failing or coming apart) was determined to be between 15 and 20 bar using
this approach (channel cross-section was 500 µm × 500 µm). Though commercial Nanoports have gained popularity as a straightforward approach to create interconnections between chips and peripheral equipment, they cannot be used for applications that require pressures above ca. 70 bar. Mair et al. reported an interface with a threaded mating port for a standard coned capillary fitting directly fabricated into an injection molded COC chip. The chip withstood 156 bar and was broken due to its delamination rather than interconnect failure. Later this approach was improved by changing the thermal fusion bonding protocol to solvent-vapour bonding and subsequent exposure of the bonded chip to UV light, to achieve a burst pressure of 346 bar. However, these authors used injection molding, which requires a new mold for each chip design and can be costly and time consuming in terms of rapid prototyping. Agilent developed a layered, polyimide microfluidic HPLC chip which incorporated an enrichment column, a packed analytical column, and a nanospray emitter with a burst pressure of 200 bar. The input capillaries were filled with a slurry of particles in 2-propanol. Each capillary was then coupled to the chip and isopropanol was used to flush the slurry into the chip under a pressure of 120 bar. The chip was sealed using standard 3.2-mm diameter ring, which enables chip pressure resistance up to 200 bar. In this system, standard HPLC connectors were used. On the other hand, Chen et al. used a non-standard approach using either a stainless steel needle with or without the self-tapping thread, that was inserted into a mating hole or screwed into an inlet hole respectively. In both cases, the flat bottom needle part was directly in contact with the microchannel. Both interfaces were compatible with pressures up to 400 bar. However, a potential issue is that such an interface is not universal and thus standardized HPLC tubing cannot be used. Furthermore, during the process of inserting the needle, a substrate can easily be cracked.

Here we report the successful application of a micromilled thermoplastic-polymer micromixer at the interface of an LC×LC system for overcoming mobile-phase mismatch between dimensions. The micromixer contains array of microfabricated grooves having so-called “herringbone” or asymmetric chevron geometry. These grooves perturb the side-by-side laminar flow profile of two solutions to be mixed such that two counter-rotated vortices are established. Solution streams are thinned leading to shorter diffusion length and thus faster mixing. The mixer developed in this study had an internal volume of 4.65 µL, which is much smaller volume than conventional units have. Using a custom-designed, robust, low-dead-volume interface, a mixing chip can be directly coupled to the LC×LC equipment using standardized HPLC connectors. The operating pressure for the chip, which is clamped in a metal
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holder, is up to 200 bar. We implemented our micromilled, COC microfluidic mixer in a HILIC×RP-LC system. When our mixer was used to incorporate make-up flow, an improved separation was obtained compared to the system with no make-up flow, as expected. We successfully identified various oligomeric series in nylon (Polyamide 46) samples as a demonstration of the 2D-LC system with implemented micromixer.
Material and Methods

Chemicals and reagents

All chemicals were analytical-reagent grade. Fluorescein was purchased from Sigma-Aldrich (NL) and used to prepare separate 5.0 μM fluorescein solutions in 10.0 mM phosphate-buffered saline, pH 7.4 (PBS; Gibco, UK). The pH was measured using pH-indicator strips (Neutralit, MERCK). All solutions were prepared with 18 MΩ-ohm ultrapure water (Arium 611, Sartorius Stedim Biotech, Germany).

Formic acid (FA) and mass-spectrometry-grade acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Milli-Q grade water (Merck Millipore system 0.22 μm) was used for preparation of mobile phases. Acetone (BASF, Ludwigshafen, Germany) was used for the dispersion test.

A list of the chemicals (and suppliers) used in this study is reported in the Supplementary Information (SI).

Fabrication of the COC chip

COC substrates (grade TOPAS 5013L-10) were purchased from Kunststoff-Zentrum (Leipzig, Germany). This grade was chosen to minimize occurrence of burrs (raised edges or small chips of plastic that remain attached to the workpiece) during the milling process. A device consists of a top and a bottom plate which are 2 mm and 5.5 mm thick, respectively. All polymers were used as received in plates and cut to the desired dimensions in-house (30×58 mm). Channels with grooves were designed in SolidWorks (Waltham, MA, USA), and the G-Code was generated from these designs for the milling machine (Autodesk, San Rafael, CA, USA). The actual groove and channel dimensions may be found in Figure 3 (Results and Discussion section). The bottom part of the chip was micromilled using a computer-numerically-controlled (CNC) micromilling robot (Mini-Mill/3, Minitech Machinery, Norcross, GA, USA) equipped with a brushless electronic Astro-E500Z spindle (NSK, Schaumburg, IL, USA) with a maximum rotational speed of 50,000 rpm. MACH3 CNC was used as control software. The channel containing grooves was milled using 2-flute endmills of 200 μm and 100 μm, respectively. Several bottom parts were milled with only a channel, as test pieces for establishing the optimal bonding conditions. The spindle speed was set to 15,000 rpm. It took 1 hour for the milling of the channel and 5 hours to mill grooves. In order to prevent
melting during milling due to the heating of the substrate, a cooling liquid (water with soap) was continuously flushed over the COC surface.

The cover plate was polished with a PICOMAX 20 machine (Fehlmann, Seon, Switzerland) to obtain better flatness for bonding later on. Conical access holes were drilled into this piece using a high-speed custom step drill. Prior to bonding, the cover plate and the channel chip were thoroughly cleaned with deionized water and isopropanol, and dried under a N\textsubscript{2} stream. The plates were then exposed to the vapour of the cyclohexane for 8 min at room temperature (COC activation). For this, plates were placed over a square glass reservoir (appr. 20×10×5 cm) filled with approximately 200 mL of cyclohexane inside a closed container. Afterwards, the top and bottom plates were aligned using 1.59 mm (1/16") polyether ether ketone (PEEK) tubing (Kinesis, Altrincham, Cheshire, UK). Two extra holes (1.6 mm diameter) were drilled on the opposite side of both plates for this purpose. An assembled chip was placed between the two chucks of a hot embosser (Dr. Collin, Ebersberg, Germany). The device was first heated from 25°C to 120°C in 20 min, then compressed with a force of 15 bar (377 N/cm\textsuperscript{2}) for 15 min at 120°C, and finally passively cooled down to 25°C.

To evaluate bonding strength, each chip was clamped in the metal holder (see following section) and the inlets were connected to HPLC pumps (Waters 515, Waters, Milford, MA, USA). An HPLC column (HYPERSIL SAX 5U 4.6×150 mm, Alltech Associates/Applied Science, Carnforth, UK) was used as a restrictor at the outlet, and water was pumped through at a total flow rate of 0.5 mL/min. After a linear increase in pressure, a sudden pressure drop was observed, indicating the burst pressure of the device.

**Micro-to-macro interface**

There are a few important requirements for the macro-to-micro interfaces of microfluidic chips, which should be met in order to be applicable in LC: they should be compatible with standardized connectors for easy implementation, provide adequate pressure stability and not introduce dead volume.\textsuperscript{36}

In order to connect the micromixer to other instruments, a specially designed micro-to-macro interface was developed (Fig. 1). The chip was clamped between two brass parts that were screwed together. For the creation of a female connector, a conical hole in the shape of an HPLC ferrule (ca. 0.5 mm) was drilled into the top COC plate and a standard 10-32 threaded hole was fabricated in the metal top part (8 mm diameter) (Fig. 1A). The bonded COC chip and
top part of the metal holder were aligned using small pieces of 1.59 mm (1/16") PEEK tubing, inserted through the standard 10-32 holes in the top metal part and into the conical holes in the device to center them around the conical holes drilled into the top of the COC device. The chip was then sandwiched between top and bottom metal plates, and the metal plates clamped together at the sides with 6 metal bolts (3 down each long side of the holder) (Figure 1B, 1C). For experiments, PEEK tubing (1.59 mm o.d., 0.254 mm i.d.) was inserted through connector holes and standard HPLC fittings were twisted into the connector holes until they were finger tight in the metal holder.

![Diagram of microfluidic setup](image)

**Figure 1.** Micro-to-macro interface for establishing the connection with LC×LC: (A) side view of the COC device aligned with top and bottom brass plates of the holder; holes for the female connectors were drilled partly in the top COC plate (conical part) and in metal (10-32 thread). (B) side view and (C) top view of the fully assembled chip clamped in the metal holder with finger-tight HPLC fittings, fixed into the assembly.

Standardized HPLC finger-tight fittings consist of a male nut (10-32 thread) with conical ferrule and female tapered ferrule seat. Because COC substrate was not commercially available in thicker plates (more than 10 mm), the fabrication of full female tapered ferrule seats inside COC top plates was not possible. We decided to fabricate only the conical part for ferrule-and-pilot depth in the COC top plate. The rest of the female connector – suitable for 10-32 thread - was fabricated in the metal top part (Figure 1A). Dimensions that were used for the fabrication of the female tapered ferrule seat are in agreement with LC industry standards.

The interface was designed in such a way that HPLC tubing was pushed directly down onto the channel in the COC chip, and the standard HPLC fittings were introduced and turned into the metal top part until finger-tight. This design is meant to minimize the dead volume in the interconnects. Such a macro-to-micro interface allowed direct connection of the
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Microfluidic chip to the LC×LC using standard HPLC fittings. Moreover, clamping between metal plates adds extra pressure stability to the chip itself. The metal holder is reusable and can be used every time with a new chip. Fittings can be inserted easily, without extra force.

In order to evaluate the pressure stability of the connections and to measure the maximum burst pressure, each inlet was connected to a UHPLC pump (Agilent 1290 Infinity, Agilent, Waldbronn, Germany) and the outlet was connected to a restrictor (GL Sciences, Eindhoven, The Netherlands, Inertsil ODS-3 column; 250 mm × 3 mm i.d., 3-µm particle size). The total flow rate was set to 0.5 mL/min and the pressure profile was recorded. In the first pressure test, the pressure limit of each pump was set to 150 bar and PEEK tubing was used. A second pressure test was performed using stainless UHPLC tubing. The pressure limit of each pump was now set to 200 bar. The chip was connected as explained above.

**LC×LC setup and chromatographic conditions**

The 2D-LC instrument in which the micromixer was tested was an Agilent Technologies Infinity 1290, consisting of two binary pumps (G4220A and G4220B) (one for each column), an autosampler (G4226A) with thermostat (G1330B), a thermostatted column compartment (G1316C) with 2-position/4-port-duo switching valve (G1170A) equipped with two 80-µL loops (Agilent, 5067-5426), and a UV detector (G4212A). The HILIC separation were carried out using an Agilent ZORBAX Rx-SIL column (150 mm × 4.6 mm i.d., 5-µm particle size, Agilent, Wilmington, DE, USA). In both HILIC and RP dimensions, water containing 0.1% FA and 100% ACN were used as eluent components. These solvents were pumped in different ratios using a 1D pump and a 2D pump. We performed 1D gradient separations at 30 µL/min using the following program: 0 min 99% ACN, 500 min 5% ACN, 510 min 5% ACN, 512 min 99% ACN, then kept at the initial solvent composition for 88 min.

The second-dimension separation column (Waters Cortecs C18, 30 mm × 4.6 mm i.d.; 2.7 µm particles) was operated at 3 mL/min at 40°C. The following gradient was used: 0 min 1% ACN, 0.3 min 30% ACN, 0.36 min 70% ACN, 0.39 min 1% ACN. The modulation time (the time between valve switches) was 0.6 min (36 sec).

The operating principle of the interface between the dimensions was as follows (Figure 2A). The microfluidic mixer containing mixes 1D effluent (30 µL/min) with a 0.1% FA aqueous solution (200 µL/min) using the make-up-flow pump (Gilson 305, Middleton, Wisconsin, USA). The mixed solution was introduced to an 80-µL storage loop (Loop 1). Each loop was
filled 100%, but some part of effluent goes to the waste. The contents of Loop 1 are transferred with the flow from the 2D Pump to the 2D column upon switching the valve (Fig.2B), while Loop 2 is being filled. This occurs in multiple alternating cycles during the whole 1D chromatographic run.

During the sequence, one blank sample (Formic acid solution) was injected after each sample, and no significant carryover of sample was observed. LC×LC separations and other tests were performed in triplicate.

All oligomers were identified manually based on high-accuracy mass-to-charge \((m/z)\) values measured by Q-ToF MS.
Sample Preparation

In the HILIC-UV experiments, 250 mg of Polyamide 46 (PA46) were dissolved in 25 mL of formic acid. The volume of injected PA46 sample was 2 µL (20 µg of PA46). The polyamide solution was injected directly into the LC×LC system without additional sample preparation.

Dispersion tests

Dispersion tests were performed using either 1) a zero-dead volume union (316 Stainless Steel, Swagelok, Solon, OH, USA); 2) a T-piece (316 Stainless Steel, Swagelok); 3) a 10-µL S-mixer (Ultra HPLC, binary input housing, 316 stainless steel, Analytical Scientific Instruments, Richmond, CA, USA); 4) 150-µL S-mixer (Standard HPLC, binary input housing, 316 stainless steel, Analytical Scientific Instruments); or 5) our microfluidic mixer with herringbone grooves. A zero-dead-volume union was placed between an injector and a UV detector (Agilent Technologies, G4212A) instead of the 2D column. The inlets of each mixing units were connected to an injector and an additional pump, and the outlet was connected to the same UV detector. For all experiments, 1 µL of a 1% solution of acetone was injected at a total flow rate of 0.5 mL/min. A concentration profile was recorded with a photodiode array UV detector at λ = 275 nm (the wavelength at which the acetone exhibits the maximum absorbance).³⁸

Mass Spectrometry Parameters

The outlet of the LC×LC system was coupled online to an Agilent 6540 UHD accurate-mass quadrupole time-of-flight mass spectrometer (Q-ToF MS) equipped with an Agilent Jet Stream ESI source (Agilent, G1958-65138) through a T-piece (316 Stainless Steel, Swagelok, Ohio, United States). The Jet Stream ESI source was operated in positive mode and instrument parameters were set as follows: gas, nitrogen; sheath gas temperature, 350°C; sheath gas flow, 11 L/min; dry gas temperature, 300°C; dry gas flow, 8 L/min; and capillary entrance voltage, 3,500 V. The Fragmentor and Skimmer were operated at 125 and 65 V, respectively. The normalized collision energy was 750 V (OCT 1RF Vpp). The scans were acquired in MS mode in a mass range from 50 to 1700 m/z at a rate of 4 spectra/s.
Data Analysis

The LC system was operated using Agilent OpenLAB CDS ChemStation Edition, version C.01.07 SRI. The LC×LC and MS were operated using Mass Hunter Workstation Software LC/MS Data Acquisition, version B.05.01 (Agilent). LC-MS data were analyzed using LC×LC Software from GC Image (Lincoln, NE, USA; GC Image LC×LC-HPMS, version R2.5b3). Quantitative evaluation of the degree of dispersion caused by the different mixing units tested was determined using a chromatogram recorded at the maximum absorbance wavelength of acetone using the Mass Hunter Workstation Software Qualitative Analysis software (version B.06.00, Agilent, 2012). The same software was applied to identify oligomeric series. All extracted ion chromatograms (EICs) were obtained with ± 20 ppm m/z expansion (the m/z value window).
Results and Discussion

Our previous research was devoted to fabrication of the HG microfluidic mixer in poly(dimethylsiloxane) (PDMS), with an optimal design for application in LC×LC. However, due to the elastomeric properties of PDMS, microchannels deformed when high flow rates are used as a result of the higher pressures induced. PDMS also exhibits poor compatibility with solvents commonly used in LC, limiting its applicability in LC. To prevent microchannel deformation, it was decided to use a more rigid material for the chip fabrication. Cyclic-olefin copolymer (COC) was the material of choice, because of its excellent optical and mechanical properties, and compatibility with the typical solvents used in HPLC. Micromilling was used as fabrication technique, as it offers a low start-up cost and a fast way to translate designs into prototypes. However, even with ongoing advancements in the technology, micromilling does have some limitations in resolution, dictated by the endmills that are used. In this work, the smallest dimension for the groove design was determined to be 100 µm (groove spacing), whereas in our earlier PDMS device this parameter was 50 µm. To retain optimized mixing performance, it was thus necessary to recalculate the other dimensions in the micromixer to accommodate larger groove widths. This was done using an approach described in the references for optimized geometries. The re-calculations were based on ratios between different channel and groove parameters according to the design protocol for optimized geometry that was described elsewhere. The dimensions that were used in this work are shown in Figure 3: the channel width and depth were set to 430 µm and 150 µm, respectively; the groove width and depth were 260 µm and 100 µm; and the ridge between grooves was 100 µm (determined by the resolution of the milling).

In order to characterize the mixing performance of micromilled COC mixers with new geometries, mixing experiments with different flow rates and ratios, including the ratio 1:7 (PBS:PBS) that was used in this work in the LC experiments, were performed (Suppl. Fig. 1). At this ratio with the total flow rate of 230 µL/min, the COC chip showed sufficient mixing performance (see the Supplementary Information).

Rounding of the internal corners of features by micromilling is caused by the shape of the endmills used. Rounded corners have a radius equivalent to that of the endmill. Experiments with a PDMS chip that incorporated rounded features were performed in advance (see the SI,
Supplementary Figure 2), but did not reveal any substantial adverse influence on the mixing performance of these rounded groove structures.

**Figure 3.** The channel of the micromixer with herringbone grooves: (A) schematic channel side view with channel and groove dimensions; (B) photograph of the micromilled COC channel at the Y-junction and (C) schematic top view of the full microchannel.

**Optimization of the bonding procedure**

To bond the top and bottom COC plates, a solvent-vapour-assisted bonding approach was used. This method allows the direct bonding of substrates to one another without the use of additional adhesive materials added to the interface. This type of bonding is especially suited for applications that require high pressure resistance. Solvent bonding of thermoplastics takes advantage of polymer solubility in the selected solvents. Exposure of the COC surface to a vapour phase can avoid excessive solvent absorption and allow a more-controlled distribution of the solvent molecules over the polymer surface. Cyclohexane was chosen as a solvent for the bonding procedure, based on very similar Hildebrandt solubility parameters (which provide a numerical estimate of the degree of interaction between materials) of COC and cyclohexane (17.7 and 16.7 J$^{1/2}$ cm$^{-3/2}$, respectively). When the surface of the COC substrate is solvated, polymer chains become mobile and can diffuse across the solvated layer into another similarly solvated COC surface layer. This leads to mechanical interlocking of chains between the two surfaces and creates an exceptionally strong bond between the two parts.
It is known that bonding at elevated temperature can greatly enhance polymer entanglement, which can result in stronger bonding. For this reason, after exposing both top and bottom COC substrates to cyclohexane vapour and aligning and bringing them into contact, the chip was heated up to 120°C (a bit lower than the glass transition temperature, which is 134°C for this COC grade), pressed together and cooled down to room temperature. Different exposure times to cyclohexane vapour and applied forces were tested in order to find the optimal bonding conditions (Table 1). Note that only devices containing non-grooved channels were tested in this part of the study.

Table 1. Parameters that were tested for optimizing the solvent-vapour-assisted bonding procedure.

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</tbody>
</table>

After each chip was clamped in the macro-to-micro interface (as discussed below), we performed an evaluation of bonding strength. A sudden pressure drop was observed at some point during a linear increase of pressure, indicating that the burst pressure of the device had been reached. All chips were visually inspected and it was noticed that in the first four chips (Table 1) top and bottom plates were delaminated from each other. Using visualization with a food dye, we observed that the area of the delamination was inversely proportional to the exposure time to cyclohexane vapour. When the pressure reached 160 bar during the experiment with the last chip (number 5), the HPLC tubing failed (due to the pressure limitation of the PEEK tubing). A visual inspection of the chip did not reveal any delaminated or damaged areas. Thus, the following bonding conditions were used for further experiments: 8 min of exposure to cyclohexane vapour and 15 bar of applied pressure for 15 min at 120°C. Pressure tests to reveal the maximum burst pressure were performed later and described in the following section.
Macro-to-micro interface
Even though the chip is meant to be placed in the interface between dimensions (separation columns) in the low pressure region (10-30 bar), the valve switching can cause unpredictable pressure pulses. It was thus decided to test the chip for the maximum burst pressure it could withstand. During the first pressure stability test with an actual mixing device with herringbone grooves, the pressure reached 150 bar and the flow was stopped. No visual damage or leakage was observed for 15 minutes. In a repetition of this test, after a linear increase of pressure up to ~180 bar, the pressure dropped, because of leakage at the PEEK tubing. This experiment was performed with three different chips with the same outcome. No delamination of the chips was observed. Because of this it was decided to use UHPLC tubing, which can withstand much higher pressures. When the total pressure reached 200 bar, UHPLC pumps started to pump only with the flow rate needed to maintain the pressure in the systems. The pressure signal on both pumps was recorded (Suppl. Fig. 4). After 4 min a pressure drop was observed. Visual inspection of the chip revealed a crack in the COC substrate that appeared in the region of the inlets. Thus, two connector holes milled in close proximity (appr. 6 mm) of each other create the weakest point in the COC substrate.

There are a few other features of the interface that should be mentioned. In order to assemble the interface, an extra screwdriver and/or wrench for the bolts is needed. Also, precise alignment between the COC chip and metal parts is required, otherwise the two parts of the female connector will be misaligned. The current interface does not allow experiments in which optical data acquisition needs to be performed. This was not required for the present application. The mixing experiments described in Supplementary Information were performed without the metal holder, with the tubing simply inserted through the inlets of the COC chip.

Dispersion test
In order to characterize the elution profile of the micromixer was compared with those produced by other conventional (mixing) components, as well as a zero-dead volume unit. To understand the effect of the integration of the micromixer into the modulation interface, the Taylor-dispersion-Analysis was performed. A flow rate of 0.5 mL/min was chosen to allow rapid testing without introducing excessive Taylor dispersion and unnecessary pressure to the system. The pressure in the system was in the range of 36-38 bar for all mixing units.

First, to obtain the value of the dead volume of the system, either a zero-dead volume union, a T-piece, one of two S-mixers (10 µL and 150 µL) or a microfluidic mixer (M-mixer)
were tested (Suppl. Figure 5). Results are shown in Figure 4. Table 2 presents the quantified data obtained from the integration of the acetone peaks in the chromatograms (n = 3).

**Figure 4.** Normalized concentration (UV) profiles for 1% solutions of acetone at a total flow rate of 0.5 mL/min for the zero-dead volume union, T-piece, microfluidic mixer (M-mixer), 10-µL S-mixer and 150-µL S-mixer. These profiles were recorded using UV detector.

As was expected, band broadening appears for all mixing units to a different extent. Among all mixing units, the least dispersion is observed in the T-piece. This can be explained by the simple construction of the T-piece, which represents a T-junction (with 0.28 µL dead volume). The geometry of the M-mixer and S-mixers are more complicated, which leads to a longer fluid path. Besides, they have larger internal volumes compared to the T-piece.

**Table 2.** Chromatographic data obtained from the integration of the acetone plug profile during dispersion tests; experiments performed in triplicate; an average value is presented.

<table>
<thead>
<tr>
<th>Unit</th>
<th>Peak height, mAU</th>
<th>Area under the peak</th>
<th>Peak width, s</th>
<th>Total elution time(^{1}), s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-dead volume union (0.007 µL)(^{45})</td>
<td>89.3 ± 0.22</td>
<td>226.8 ± 2.76</td>
<td>5.4 ± 0.01</td>
<td>6.0</td>
</tr>
<tr>
<td>T-piece (0.28 µL)(^{46})</td>
<td>79.2 ± 0.56</td>
<td>226.7 ± 2.2</td>
<td>7.5 ± 0.01</td>
<td>9.0</td>
</tr>
<tr>
<td>M-mixer (4.65 µL)</td>
<td>65.6 ± 0.62</td>
<td>222.8 ± 5.9</td>
<td>9.0 ± 0.02</td>
<td>10.8</td>
</tr>
<tr>
<td>S-mixer (10 µL)</td>
<td>62.5 ± 1.18</td>
<td>222.7 ± 7.5</td>
<td>9.6 ± 0.06</td>
<td>12.0</td>
</tr>
<tr>
<td>S-mixer (150 µL)</td>
<td>11.0 ± 0.30</td>
<td>213.8 ± 9.5</td>
<td>49.8 ± 0.10</td>
<td>58.8</td>
</tr>
</tbody>
</table>

\(^{1}\) The elution time from the beginning of acquisition till the last point of the elution of each peak profile on the chromatogram.
M-mixer with the inner volume of 4.65 µL yields a concentration profile similar to the S-mixer with the volume of 10 µL (the peak width of the S-mixer is slightly larger due to tailing). The similarity of the M-mixer to the mixing unit with a larger volume can be explained by the location of the transverse fluid transport caused by grooves in M-mixer. Mixing occurs both above and within the grooves. While rapid chaotic mixing occurs above the grooves due to vortex generation, mixing within grooves is governed by laminar flows and slow diffusion. Thus, the molecules that enter a groove spend a longer time in the channel than molecules that remain in the open channel, resulting in increased dispersion. Acetone elutes from the micromixer over a time period of 10.8 s compared to 6 s with a zero-dead volume union. However, the microfluidic mixer provides much lower dispersion than the 150-µL S-mixer (dispersion peak width of ~ 50 s), which is recommended for the flow rate range that is used in our work (100-250 µL/min). From this perspective, the developed microfluidic mixer provides a very good alternative to the S-mixer in LC×LC under these flow rate conditions.

**Online LC×LC separation of Polyamide 46**

Polyamide 46 consists of linear and cyclic oligomers of adipic acid (ADP) and 1,4-diaminobutane (DAB).

To demonstrate the use of the M-mixer in 2D-LC, we focused on the identification of the four most important oligomeric series: ADP-(DAB-ADP)$_n$-2-DAB-ADP, cyclic oligomers, ADP-(DAB-ADP)$_n$-2-DAB and DAB-(ADP-DAB)$_n$-2-ADP-DAB. HILIC and RP were chosen for the LC×LC separation in the first and the second dimensions, respectively. In order to minimize eluent mismatch between these dimensions, a modulation interface (Figure 2) consists of a two-position 8-port switching valve equipped with two loops, a mixing unit and an additional pump were used. In this approach, a make-up flow is used to reduce the mobile-phase strength of the 1D effluent on the 2D column using a microfluidic mixer with herringbone grooves for this purpose.

To develop the LC×LC separation, we optimized each dimension separately. Afterwards, a HILIC×RP-LC separation of Polyamide 46 without make-up flow was performed. The results are presented in Figure 5A. When the fraction of 1D effluent with a high content of acetonitrile reaches the 2D column, a substantial portion of the sample peak is not able to interact with the stationary phase and elutes with the solvent front around the column dead-time ($t_0$ ≈ 7 s). This is the so-called “solvent-plug peak” or “breakthrough peak” (Figure 5A).

To trap oligomers eluting in an ACN-rich mobile phase, the effluent from the 1D (30 µL/min) was mixed with a make-up flow of water containing 0.1% FA (200 µL/min), diluting
the 1D eluent almost 7 times. Since the HILIC gradient in the first dimension varies from 99% to 5% ACN, the mobile-phase composition varies between 14.8% and 0.7% of ACN after dilution. The results, obtained using this approach, are shown in Figure 5B. No breakthrough is observed and good separation is obtained.

Due to the 7.67-time dilution and the fact that some of the sample went to the waste in the experiment when a make-up flow was used, the absolute amounts of injected sample on the second column was lower than in experiment with no make-up flow (in the last case a loop was filled only with 1D effluent).

![Figure 5.](image)

In most cases, a dilution of the sample is seen as something to be avoided, as it can cause additional band broadening and a decrease in sensitivity. However, when used in-between dimensions, a weaker sample solvent obtained by dilution not only improves retention of analytes on the 2D column, but it also tends to limit band broadening due to absorbing analytes in a narrow zone at the top of the column, a so-called on-column sample-focusing effect.⁴
Figure 5B shows the two-dimensional separation of a Polyamide 46 sample in which four identified oligomeric series are indicated. Their chemical structures are given in Figure 5C. The elution behavior of these series obeys simple rules related to the chain length, where the monomer elutes first and the higher oligomers elute successively in order of increasing chain length. During the HILIC separation, oligomers elute based on their chemical composition. On the one hand, the retention increases with increasing sample polarity and the increase in number of polar functional groups in the molecule usually enhances sample retention. On the other hand, the interactions of basic and acidic analytes with the stationary phase are expected to be based on both hydrophilic interactions and electrostatic forces. The elution sequence on the HILIC column can be explained by the ionized state of the primary end groups. It is believed that electrostatic interactions play an important role in HILIC, due to the partially ionized residual silanol groups on the surface of the silica stationary phase. This leads to a negatively charged surface that creates an electrostatic field in the contacting mobile phase. In the case of the ADP-(ADP)$_n$-ADP series, the weak retention in the first dimension can be explained by repulsion between the silica gel surface (which is negatively charged due to the adsorbed water molecules and carboxylic-acid end groups that are partially negatively charged due to the presence of water (absorbed layer on the stationary phase). However, the main fraction of carboxylic-acid end groups remain neutral due to the formic acid that is present in the solute plug. For the cyclic series, which has no terminal functional groups, the slightly better retention can be explained by other polar interactions, such as hydrogen bonding and dipole–dipole interactions. In the oligomeric series ADP-(DAB-ADP)$_n$-DAB, which contains both a carboxylic-acid group and a primary amine group, the retention mechanism is a superposition of electrostatic interactions and hydrogen bonding to the stationary phase. The last identified series was DAB-(DAB)$_n$-DAB, which contains only amine end groups that may be partially protonated in the presence of formic acid. These molecules have the highest retention on the HILIC column due to the attractive electrostatic interactions. Due to good retention, these compounds stay the longest in the first dimension, which results in a very broad peak shape, long tailing and a large number of modulations required to transfer one-dimensional peaks to the second dimension.

Besides these identified series, other oligomeric series are visible in Figure 5B (not marked) (e.g. with adipic acid/pyrrolidine, adipic acid/amide and 1,4-diaminobutane/amide end groups). However, we focused only on the identification of the main four series discussed above.
Oligomers with carboxylic-acid end groups are weakly retained on HILIC and are eluted in the beginning of the gradient at higher concentrations of acetonitrile. The utilization of the microfluidic mixer for dilution of 1D effluent before the second dimension allows the first series of oligomers (ADP-(ADP)_n-ADP) to provide some focusing effect on the 2D column. Figure 6 shows extracted-ion chromatograms (EICs) for ADP monomer (Figure 6A) and tetramer ADP-(ADP)_2-ADP (Figure 6B). Each peak represents one second-dimension run (60 s) from one collected fraction and the analyte is distributed over these chromatograms. As can be seen from Figure 6A and 6B, each first-dimension peak was sampled 9-10 times, resulting in the 9-10 peaks shown. The obtained 2D chromatograms are sharp. However, a bit of tailing can be observed, the cause of which is not completely understood, but may be related to a lack of the complete focusing at the top of the column for the early eluting analytes. With each succeeding oligomer in the series the tailing becomes less, to disappear completely for tetramers (Figure 6B).
Oligomers with primary amine end groups (DAB-(DAB)n-DAB) are strongly retained on the HILIC column due to attractive electrostatic interactions between partially negatively charged silica gel surface and partially protonated amino groups. This series exhibits the most band broadening during the first-dimension separation and it is the most affected by the intermediate addition of water (aqueous buffer). Figure 6C shows the EIC for the DAB monomer. This 1D peak is very wide, has substantial tailing and takes more than 29 modulations to be transferred to the second dimension.

In order to compare the performance of the M-mixer with other mixing units, we conducted experiments with a T-piece and two S-mixers (10 µL and 150 µL). Although the overall performance of all mixing units was similar, several effects were observed. We noticed a shift to longer total elution times for all mixers with respect to the T-piece. This shift was small for the 10-µL S-mixer and larger for the M-mixer. A clear pattern for the shifted elution times of the M-mixer starting from 2 min for ADP monomer (Fig. 7) up to 7 min for DAB monomer (Fig. 8) was observed. The delay in elution can be caused by the change in 1D flow rate when a mixing unit is placed directly in the fluid flow and acts as a restrictor. In the case of S-mixers the restriction is small because the inner cartridge has a bigger diameter compared to the long narrow channel of the M-mixer. However, a delay of 7 min causes only a 1%
increase in the total analysis time. Furthermore, the quality of 2D separation, when the M-mixer is used, is not influenced by this shift.

![Figure 8](image)

**Figure 8.** Extract ion chromatograms (EICs) of the DAB monomer (m/z value is 287.24) obtained with (A) T-piece, (B) 10-μL S-mixer, (C) 150-μL S-mixer and (D) m-mixer in the interface of 2D HILIC×RP-LC system for analysis of PA46 sample.

We also noticed that the 150-μL S-mixer, which is recommended by the manufacturer for the flow-rate range 100-250 μL/min, gave rise to split peaks for the first oligomeric series (Supplementary Figure 6C), for which the mixing is most important. This can be caused by incomplete mixing and the distribution of sample between different regimes having different compositions. Additionally, the number of modulations required to transfer one 1D peak was higher than for any other mixing unit, due to broadening of the peak. Finally, the volume of this mixer is almost twice the volume of the loop size. In this case, resolution is lost inside the mixer itself due to its relatively large volume.
Supplementary Information

Characterization of the degree of mixing
For characterization of the degree of mixing, fluorescence detection was used. Fluorescein (5 μM) in phosphate buffer (pH 7.4) was introduced from one inlet whereas 10mM phosphate buffer was introduced from the other inlets into the Y-junction of the channel at different flow rates using syringe pumps with 5-mL syringes (Prosens NE1000, The Netherlands). The chip was placed on an inverted fluorescence microscope (model “DMIL”, Leica Microsystems, The Netherlands), equipped with a 4×objective, an external light source for fluorescence (EL6000, Leica Microsystems, The Netherlands), and a CCD camera. For visualization of fluorescence, a fluorescein filter set (488 nm excitation, 518 nm emission) was used. Images were captured at different positions along the channel with a CCD camera connected to a computer using a 4x objective magnification with a field of view of 1.8 mm, a 1-sec exposure time, a gamma setting of 1.75, and a gain of 3.5.

The mixing performance of COC-milled micromixer
After the COC chip was micromilled and assembled, its mixing performance was tested at different flow rates. The protocol for testing the mixing efficiency was described in our previous work.\textsuperscript{26}

The degree of mixing was quantified by determining the standard deviation (SD) in fluorescence intensity across the width of the channel at different locations along the channel length. The detailed data analysis procedure described elsewhere.\textsuperscript{26} For the purpose of this study, sufficient to say that high SD in fluorescence intensity across the mixing channel correlates with incomplete mixing. The degree of mixing is inversely proportional to SD. The high SD is measured at the very beginning of the mixing channel (at the Y-junction), where the two solutions (5μM fluorescein solution in PBS buffer and PBS buffer) meet and enter the channel under laminar flow conditions (no mixing).

For the purpose of mixing in the interface between two dimensions of LC×LC to adjust mobile-phase compositions, it was import to test different flow rate ratios as well. Supplementary Figure 1 shows results for the mixing performance experiments with different flow rates at 1:1 ratio and with the flow rates used for this work at 1:7 ratio. As usual, the mixing efficiency increases (standard deviation, SD drops) toward the end of the channel. It was observed that when the ratio of inlet flows was 1:1, mixing behaviour was similar for all tested
flow rates. However, the mixing is not complete by the end of the 40-mm channel. The flow rate ratio 1:7 (30 µL-min – 200 µL-min) was important to test because that was the flow rate ratio that we wanted to use further in the application of the mixer. Slightly deteriorated situation was observed, which can be explained by the difficulties to involve thinner fluid layer into the general chaotic flow. Similar situation was observed in our previous research too. Nevertheless, the COC chip showed sufficient mixing performance for all flow rate ratios.

Supplementary figure 1. Mixing efficiency in the COC-micromilled microfluidic mixer at different flow rates and different flow ratios (1:1 and 1:7); 5 µM fluorescein in PBS mixed with PBS; channel width 430 µm; channel depth is 150 µm; groove depth is 100 µm; the total channel length 40 mm.

Experiments with groove rounded angles
The radius of the endmills, used for the micromilling process, do not allow for the creation of sharp concave corners. All concave have an internal radius of curvature depending on the endmill used. Previous experiments reported by Stroock and showed that herringbone grooves with an intersection angle $\theta$ of 90°, the angle between long and short groove arms (Supplementary Figure 2A), results in the generation of maximum transverse flows. In order to investigate how the geometry of the grooves with rounded concave corners affects mixing, chips with grooves having this geometry were designed and replicated in PDMS. A detailed fabrication procedure can be found in our previous report. Only one angle in the groove design
was not rounded (Supplementary Figure 2B). This angle is an outer angle and was expected to stay sharp during the micromilling.

Supplementary figure 2. (A) Schematic drawing of grooves in a channel with herringbone grooves showing long and short groove arms and groove intersection angle $\theta$. (B) Microscope images taken from above the microchannel with herringbone grooves that have rounded angles. Only one angle of the groove was not rounded (marked in red).

Supplementary figure 3. Efficiency of mixing in the PDMS microfluidic mixer with rounded angles herringbone grooves at different flow rates at 1:1; 5 µM fluorescein in 10mM PBS (pH 7.4) mixed with PBS; channel width 430 µm; channel depth is 150 µm; groove depth is 100 µm; n=3 chips, the total channel length 45 mm.

Supplementary Figure 3 shows that SD levels, which implies that the mixing is complete, after 20 mm of the channel length at all flow rates. Here, an increased optimized dimensions comparing to our previous work were used but obtained results were similar. Thus, we decided to proceed with the fabrication by micromilling of a microfluidic mixer based on this design (dimensions, for eventual use under high pressure).
Microfluidic micromixer as a tool to overcome solvent incompatibilities in two-dimensional liquid chromatography

**Pressure test**

Supplementary Figure 4 shows the results for the second pressure test that was performed with a COC chip containing a grooved channel (dimensions are given in Fig.3). The chip withstood 200 bar for 4 min.

![Supplementary figure 4. Pressure test with microfluidic COC chip: (A) position of the chip between two UHPLC pumps as also given in Fig.2 both schematically and in a photo and (B) pressure profile for each pump (red and blue signals). After 4 min at 200 bar, a pressure drop was observed due to the crack at the inlets.](image)

**Tests with different mixing units**

In order to investigate the influence of the mixing in the interface between two dimensions, microfluidic mixer (m-mixer), 10-µL S-mixer, 150-µL S-mixer and T-piece were tested. Supplementary Figure 5 presents the different mixing components that were tested to obtain the value of dispersion of the system.

![Supplementary figure 5. Units that were used for dispersion test: (1) 4.65-µL microfluidic mixer (M-mixer), (2) 150-µL S-mixer, (3) 10-µL S-mixer and (4) T-piece and (5) a Zero-Dead Volume Union.](image)
Conclusions

In this work, the possibility to use microfluidic technology to improve LC×LC separations has been successfully demonstrated. We fabricated a microfluidic mixer with herringbone grooves that was implemented in a HILIC×RPLC system for successful separation and identification of various oligomeric series in Polyamide 46 samples. A microfluidic chip was fabricated in rigid COC substrate, which has excellent properties for chip-based LC technologies. Thanks to the solvent-vapour-assisted bonding approach, a strong bond between two COC parts was obtained. The resulting chips, when clamped in a metal holder, are able to withstand pressures up to 200 bar.

In order to create a connection with an LC×LC system, a very robust, low-dead-volume interface was developed. Using standard HPLC connectors, the chip can be directly coupled to any LC equipment. This opens a perspective for the future implementation of the microfluidic components in conventional instrumentation for improved performance, even at high pressures.

The microfluidic mixer successfully coped with the mobile-phase mismatch between two dimensions, diluting the 1D effluent before transferring it to the second dimension. As was shown, good mixing prevents breakthrough and gives good peak shapes in the second dimension. Due to the small size and ease of design adjustment, the developed microfluidic micromixer can be integrated with trap columns on one chip in order to provide an active modulation in the LC×LC interface. This can be an attractive solution to improve LC×LC modulation even further, applying modulators with smaller inner volumes with the capability to adjust mobile-phase compositions much more rapidly and efficiently.
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