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Keywords

Real-time monitoring; microfluidic set-up; mass spectrometry; microreactor; on-line coupling

Abstract

Monitoring in real-time a synthesis reaction could allow not only the detection of the intermediates involved in the synthesis to better understand its mechanisms but also the impurities. Spectroscopic methods could be performed but are not so performant when analyzing complex mixtures and could require specific properties for the detection of the molecules of interest, the presence of a chromophore moiety for example. Mass spectrometry (MS) may overcome these limitations and is able to reach the accuracy and sensitivity required to efficiently detect, quantify, identify and characterize the reagents and species produced during the synthesis. This is why the hyphenation of a microreactor with MS has already allowed to monitor synthesis processes, but most of the time targeting a specific reaction or compounds and involving solvents compatible with MS. In this study, a universal set-up hyphenated a microreactor with MS and based on two valves has been developed. This two-valve set-up has proven itself for the analysis of molecules of different nature and hydrophilicity, soluble in a large number of solvents even in non-MS-compatible ones. The developed set-up evidenced a good repeatability and a linear response for the detection of the studied compounds. In addition, the dilution step included in the two-valves set-up allows MS monitoring of compounds initially synthesized at different concentrations. Finally, it was successfully used to study an amination reaction allowing the detection of the reaction products in 4 min with good repeatability as RSD values of MS signals were lower than 17%.

Introduction

Most chemical reactions undergo a complex sequence of steps by means of reactive intermediates. The detection of these intermediates is essential for elucidating and understanding the mechanism of a synthesis reaction in solution (1). The investigation of such dynamic processes has traditionally relied on spectroscopic methods, which require the presence of chromophores, fluorophores, radiotracers, or nuclei with proper spin characteristics. The accurate determination of the rate of consumption or formation of species involved in a typical reaction requires that the chromophores or tracers undergo sufficient changes during its progress, or that the different species can be isolated to allow for their explicit identification and quantification. For this reason, spectroscopic methods have huge limitations when the monitored species of interest are present in complex mixtures, the sample availability is scarce, or the accumulation of short-lived intermediates is limited (2). Alternative approaches have been explored to overcome these limitations. Mass spectrometry (MS) is particularly well suited to the characterization and quantification of analytes present in trace amounts in complex mixtures, which can be obtained with an accuracy, sensitivity, and speed that spectroscopic methods cannot match. However, real-time monitoring of a chemical reaction involves coupling the reactor directly to the mass spectrometer, to follow the disappearance of the reactants, to confirm the presence of the desired product(s), to identify intermediates and impurities, to accelerate the optimization of the process and finally to determine the completion point (3).

The synthesis reactions are conventionally carried out in batch, which requires lab scale equipment that in turn requires the use of large volumes of samples and reagents with increased handling hazardous materials (4). However, it is now possible to develop continuous flow chemical reactors. These innovative and more environmentally friendly reactors are attractive because of their ability to mix reagents rapidly, to provide homogeneous reaction environments, to vary reaction conditions continuously, to add reagents at specific time intervals during a reaction, (5). Moreover, continuous flow chemical microreactors (µRs) have been increasingly adapted for many reasons, such as their suitability for dangerous reagents and extreme temperatures, as well as ease of scale-up, on-demand production of precise required quantities, improved throughput, and decrease in the amount of reagents (6). Therefore, the development of methods allowing the direct monitoring by MS of µR content is therefore essential (6). To be robust, the coupling of a µR with an MS detector for real-time monitoring requires designing adapted set-ups allowing high adaptability (concentration level of reagents, nature of the reaction solvent, nature and number of monitored compounds…) while maintaining the performance of the mass analyzer.

Different studies related to the use of MS to monitor a chemical reaction have already been published (1,3,7–17) (see **Table S1** in supplementary materials). Most of the oldest studies involved directing the entire flow from the µR directly into the MS ionization source (1,7–9). Some of these studies described the use of solvents, such as dichloromethane (DCM) or toluene, which are not recommended in MS as they are too volatile to be infused at 100% in the ionization source, but which were sufficient for the identification of reaction products, their quantitation of being not

the objective of these studies. The introduction of a single T-piece to dilute the µR effluent with an MS-compatible solvent (10) and of a second T-piece allowing to split the effluent and then decrease the flow-rate entering the ionization source was also proposed (3,11,12,15–17). Even if the objective was only a qualitative monitoring of the produced compounds, one of this study showed that the composition of this MS-compatible solvent (proportion of acetonitrile (ACN) in water and nature of the acid additive) strongly affects the MS signal of the monitored compounds (3).The dilution of the whole µR effluent was also achieved by digital microfluidic by merging droplets (14).

Other studies consisted in developing devices to transfer to MS only a fraction of the reactor content with a solvent chosen for its miscibility with the synthesis solvent and its better compatibility with MS. For this, some groups proposed to use conventional switching valves equipped with a 60 nL- (15) or a 5 µl-loop (12), whereas others used an available commercial "mass rate attenuator" (MRA, Rheodyne) device allowing to pick-up and transfer three different aliquots of a fixed volume (22, 100, and 300 nL), with switching frequencies that range from 0.2 to 2.0 Hz, using a transfer solvent thus allowing a dilution factor of 1,000 to 6,666, the resulting effluent being next split before entering into the MS ionization source (3,16,17).

In conclusion, more or less complicated setups allowing the monitoring of chemical reactions were developed. The simplest systems, without any dilution or flow splitting step, have been only applied to qualitative analysis, thus explaining the possibility to use solvents not well adapted to MS ionization (DCM, toluene) when only collision-induced dissociation experiments (CID) are expected to confirm the presence of products (1,7,9). Moreover, most of the studies reported in literature (77% of the cited works) concern qualitative or semi-quantitative analysis, the latest consisting in studying the evolution of the signal when varying some experimental parameters such as the temperature of the µR (3) or for kinetic studies (8,12) and polymer growth (11).

Concerning the two quantitative studies [15, 17], only the loop and then the dilution/divider assembly were at the miniaturized scale as opposed to the reactor in the first one (15). In the second one, the set-up was based on the commercial MRA system, which allows to transfer an aliquot of the reaction mixture to MS while ensuring a dilution step with a MS compatible solvent (17). However, the injection of the reaction mixture into the MRA system seems to have been done manually thanks to a valve equipped with a first loop introduced upstream of the MRA**.**

Thus, none of these studies really describes the direct coupling of a µR with MS allowing the quantitative analysis of the reaction medium. However, these studies underline the importance of carrying out a dilution step with a MS-compatible solvent and limiting the flow rates arriving in the ionization source by introducing a flow divider. Moreover, these studies focused on a single chemical reaction without any indication of the method's versatility and its applicability to other reactions, making them compound dependent. In addition, the vast majority of the syntheses were done with an MS-compatible solvent (MeOH, H_2O, ACN) which raises a major concern regarding the use of a broad range of non-MS compatible solvents for the synthesis such as dimethylformamide (DMF) and dimethyl sulfoxide (DMSO).

Thus, the main objective of this study was the development of a system allowing to couple a µR, working with flow rates of the order of 50-500 µL/min and supporting a pressure lower than 2 bar, on-line with MS by verifying the capacity of this device to study various reaction media in terms of nature of reagents and solvents. This set-up must allow to analyze only a fraction of the µR content thus implying to aliquot a fraction of its content whose reactants were introduced at a concentration of 0.1 M. Different acid, basic, and amphoteric compounds belonging to a wide range of polarity were then taken as model molecules to evaluate the developed set-up using different solvents often encountered in synthesis. Different set-ups integrating one or two valves with loops of different volumes and qualities were evaluated to provide a robust device allowing fast consecutive and qualitative analysis of the µR effluent.

Materials and reagents

Chemicals

HPLC-grade acetonitrile (ACN), dichloromethane (DCM), acetone, dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and ethanol were supplied by Carlo Erba (Val de Reuil, France). The ultra-pure water was obtained with a Direct-Q3 UV system (Millipore, Molsheim, France). Ammonium acetate (Am Ac) was provided by Merck (Darmstadt, Germany). Acetaminophen, aniline, acetanilide, aminophenol, valsartan, levofloxacine, lidocaine, rotigotine, rivastigmine, sitagliptine and trimethobenzamide were provided by Sanofi (Vitry sur Seine, France). Each compound solution was prepared at 50 mM with different solvents.

Apparatus

The analyses were carried out using a triple quadrupole mass spectrometer (TQ Ultivo, Agilent Technologies) equipped with an electrospray ionization source operated in the positive ion mode. The capillary voltage was set at + 5 kV, the desolvation gas temperature at 50°C, the gas flow (N_2) at 10 L min^{-1} and the nebulization gas (N_2) at 15 psi. Data analysis were acquired with a scan time of 500 ms. Rheodyne® valve with a homemade capillary sample loop of 150 nL capacity (length 7.3 cm, internal diameter 51.15 µm) and a VICI-Valco® valve (C82—6676) with a homemade sample loop of 300 nL capacity (length 9 cm, internal diameter 65 µm) were used. The flow through the apparatus was attained by 3 LC pumps: a nano-LC system (Ultimate 3000 RSLC nanosystem, ThermoFisher Scientific, Courtaboeuf, France) equipped with a loading pump, and an Agilent 1200 LC system with a G1376A cap-pump module (Agilent Technologies, Les Ulis, France). The Nano-LC system was conducted using chromeleon 7 (ThermoFisher Scientific) while the LC pump and MS data analysis were conducted using MassHunter Data Acquisition while MassHunter Qualitative 10.0 program was used for data treatment, both provided by Agilent Technologies. The microreactor used for coupling reaction and analysis consists in a PFA tube of 1/16” outer diameter and 1/32” inner diameter with 500µL internal volume. The inlet is connected to a Y-junction where the reactants injected by two syringe-pumps (New Era Pump Systems NE-1000) meet.

Results and discussion

1- Choice of the model molecules and solvents

Different acid, basic, and amphoteric compounds belonging to a wide range of polarity and listed in **Table 1** were taken as model molecules to evaluate the developed set-up. Their structures are provided in **Fig. S1.** These model molecules are not only mainly pharmaceutical drugs but also reagents involved in the synthesis reactions of acetaminophen and of acetanilide taken as model reactions in this study (**Fig. S2**). Because of their wide range of polarity, different solvents, often involved in chemical reactions, were also integrated in this study to test different compound/solvent combinations. Those solvent are: dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetonitrile (ACN), dichloromethane (DCM), ethyl acetate, methanol (MeOH), dioxane, toluene, ethanol (EtOH), and water. Some data (polarity index, solubility, miscibility with some MS-compatible solvent…) related to these solvents are provided in **Table S2**. This table strongly illustrates the poor solubility of some solvents with water or a mix of water and acetonitrile that are both fully MS-compatible solvents. It also shows the very poor MS-compatibility of some of them as DMSO and DMF, thus illustrating the challenge of this project.

Table 1: Selected model molecules with their monoisotopic mass, formula, log P and pKa values (source: PubChem or Drugbank). ac: acid pKa, b: base pKa.

Compounds	Monoisotopic mass (g/mol)	Formula	Log P	pKa
4-aminophenol	109.1	C_6H_7NO	0.04	5.48 (b), 10.46 (ac)
Acetaminophen	151.1	$C_8H_9NO_2$	0.9	9.38 (ac)
Acetanilide	135.1	C_8H_9NO	1.2	0.50 (b)
Aniline	93.1	C_6H_7N	0.9	4.60 (b)
Levofloxacin	361.1	$C_{18}H_{20}FN_3O_4$	2.1	6.25 (ac),8.3 (b)
Lidocaine	234.2	$C_{14}H_{22}N_2O$	2.4	7.9 (b)
Rivastigmine	250.2	$C_{14}H_{22}N_2O_2$	2.4	8.9 (b)
Rotigotine	315.2	$C_{19}H_{25}NOS$	4.7	10.97(b)/10.03(ac)
Sitagliptine	407.1	$C_{16}H_{15}F_6N_5O$	1.5	8.8 (b)
Trimethobenzamide	388.2	$C_{21}H_{28}N_2O_5$	2.3	8.8 (b)
Valsartan	435.2	$C_{24}H_{29}N_5O_3$	1.5 or 4.0	4.00 (b), 4.61 (ac)

2- Development of a dilution-MS set-up

The choice of an on-line sampling approach is critical as it specifies the transfer mode of an aliquot from the reaction system to the analytical system, MS here. The idea was to develop a set-up composed of a sampling valve with a modifiable sample loop to control the microreactor effluent volume transferred to MS and of a dilution step with an MS-compatible solvent. The expected versatility of the set-up should avoid the use of a MRA device having only 3 fixed aliquot volumes available and poor information about the generated back pressure.

With regard to the selection of the loop dimensions, it had to be done with consideration of the back-pressure generated for a given flow rate, which depends on the loop length and internal diameter. This is why experimental measurements of back-pressure were performed and are reported in **Table S3** by pumping water at two extreme flow-rate values, 50 and 500 µL/min, through 10 cm-capillaries of various internal volumes. As the maximum backpressure that the µR could support is 2 bar, for a 50 µL/min flow-rate a 65 µm inner diameter (I.D.) loop must be chosen while at least a 100 µm I.D. loop must be selected for a 500 µL/min flow-rate. Following these preliminary experiments, it was decided to develop a first set-up that could be connected to the µR via a 6-port switching valve (7010 Rheodyne) having a homemade 300 nL loop in capillary peek to maintain a pressure lower than 2 bar for an effluent arriving from the µR at a flow-rate of 50 µL/min. This valve was also connected to a LC pump that delivers a mix of ACN-water (50:50) containing 3 mM of Am Ac, *i.e.* a mix that is often used as mobile phase for LC-MS analysis as it favors ionization in electrospray. A scheme showing this primary tested one-valve set-up is presented in **Fig. 1a**, where the µR was replaced by a manual injection with a syringe for the first evaluations. A flow rate of 300 µL/min was set for quickly transferring the loop content to the MS in the set-up presented in **Fig. 1a.**

In order to test the ability of this one-valve set-up to transfer the loop content to the MS, manual injections of acetaminophen at 0.1 mM in a mix of ACN and water (50:50) containing 3 mM Am Ac, a fully MS compatible media, prepared from a 0.1 M stock solution of acetaminophen dissolved in EtOH, were carried out. Before this, the source parameters of the MS were optimized for the ionization and detection of acetaminophen by a direct infusion of the same previous 0.1 mM solution of acetaminophen prepared in ethanol. The extracted ion profiles (EIPs) of the acetaminophen analyzed in triplicates with the set-up of **Fig. 1a** are presented in **Fig. 2a.** The relative standard deviation (RSD) of the peak heights (n=3) was 12.9%. This indicated the applicability of this set-up to analyze diluted samples with acceptable RSD.

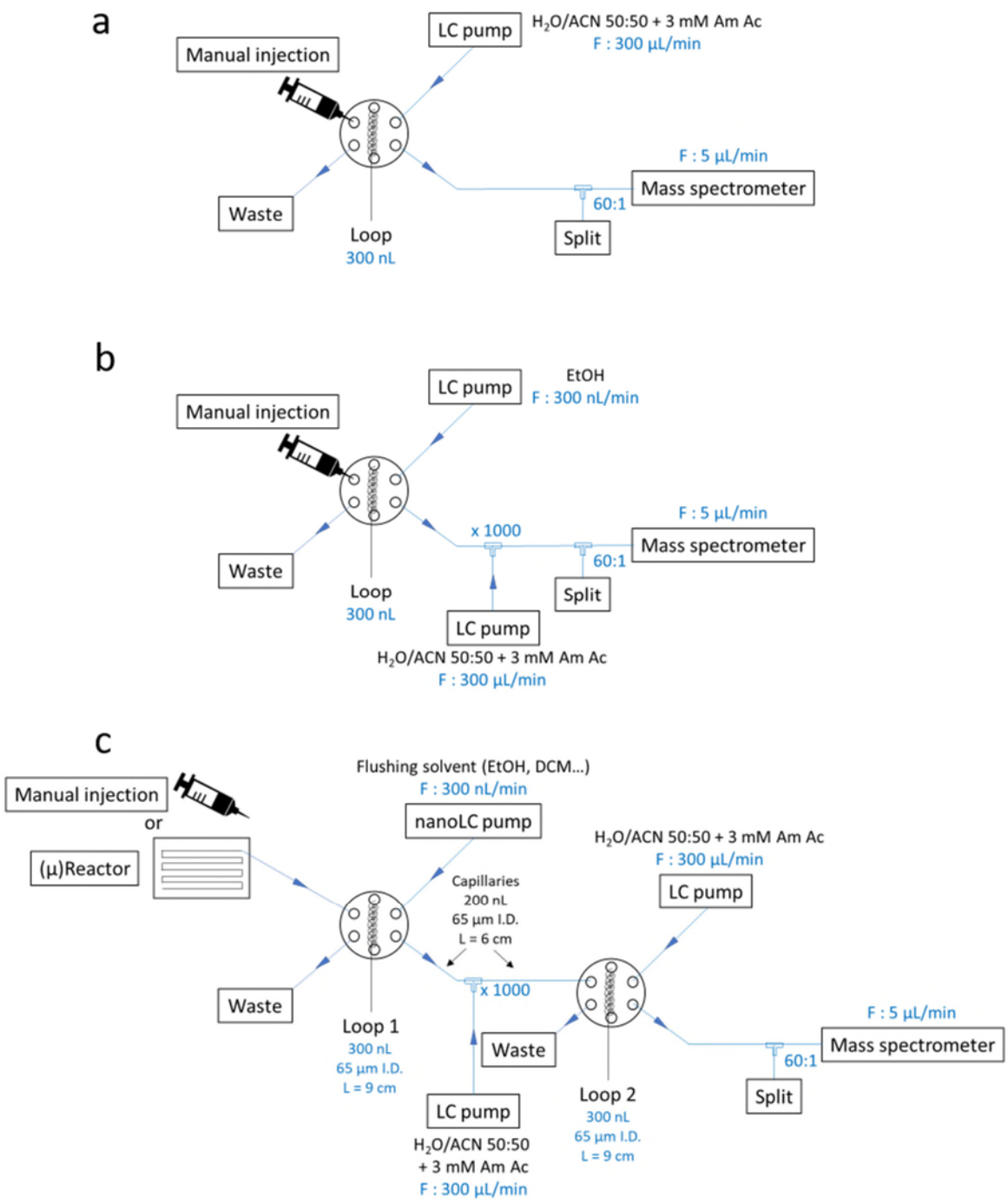

Fig. 1 Schemes representing the primary tested one-valve set-up with one (a) or two T-pieces (b) and the two-valves set-up (c). Valve from Rheodyne, 300 nL-loop in Peek.

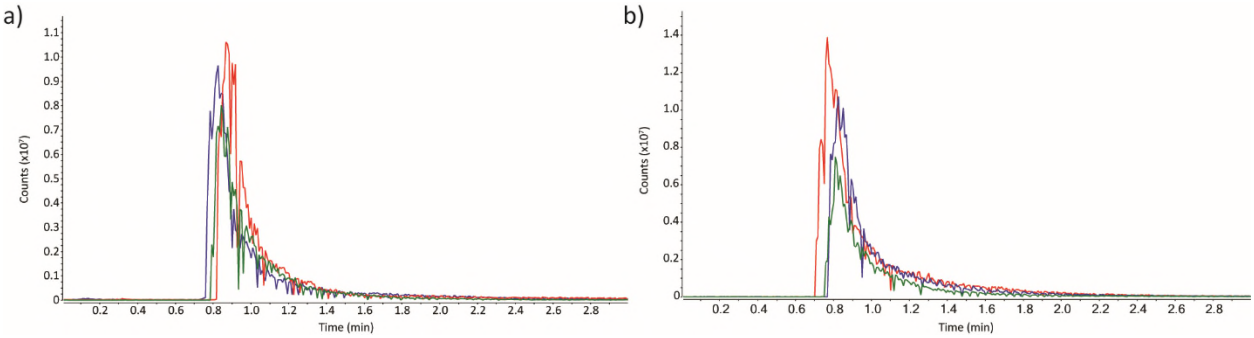

Fig. 2 Extracted ion profiles (EIPs) of acetaminophen, (*m/z* 152.1) injected (a) at 0.1 mM in a mix of ACN and water (50:50) containing 3 mM of Am Ac in the set-up presented in Figure 1a or (b) at 0.1 M in ethanol in the two-valves set-up described in Figure 1c. Each injection was done in triplicate. Valves from Rheodyne, 300 nL-loops in Peek.

Since the concentration levels expected in the µR effluent are close to 0.1 M, which is a high concentration, a second one-valve set-up was developed to add a dilution step to allow the analysis of more concentrated samples without saturating the MS detector (**Fig. 1b**). This set-up contains an additional T-piece placed between the loop and the split device to dilute the flow with the MS compatible solvent previously tested by a factor of 1000. Manual injections of acetaminophen at 0.1 M, i.e. 1000 times more concentrated than previously, were carried out and led to a carryover (**Fig. S3**), certainly caused by the limited solubility of acetaminophen together with a saturation of the MS signal when injecting acetaminophen at 0.1 M. This is why, a new set-up based on two valves was next investigated.

3- Modification of the dilution set-up: development of the two-valves-based set-up

To limit carryover, a two-valves set-up, as reported in **Fig. 1c,** was developed. In this set-up, the first valve equipped with the 300 nL-loop was maintained for its compatibility with the constraints linked to the reactor (flow-rate of 50 µl/min, limited pressure of 2 bar). This first valve is flushed by the same solvent as the one used in the µR for the chemical reactions to solve solubility problems. It allows to redirect the sample towards a dilution step (x1000) performed by a solvent with a composition similar to the one used for ESI-MS analysis. Then, this diluted sample was transferred to a second loop placed on a second 6-port switching valve before its further transfer to ESI-MS, keeping the T-piece just before the MS to split the effluent by a 60:1 ratio to fix the flow rate at the entrance of the MS at 5 µl/min.

The developed system was then tested by direct injections of acetaminophen at 0.1 M in EtOH. The first parameter to be optimized was the transfer time, which is defined as the time required for the sample to be transferred from the first to the second loop after its dilution using a flow-rate ratio of 1000. Several transfer times were tested and the best result was obtained when applying transfer time of 10 min. The corresponding EIPs obtained for the analysis of acetaminophen (0.1 M in ethanol) in triplicates are presented in **Fig. 2b.**

The comparison of the height and the area of the peak acquired with the one-valve and the two-valves set-up (**Figures 2a** and **2a**) are reported in **Table 2** along with the results acquired in the

same condition for acetanilide (another tested molecule injected at 0.1 M in DCM and in triplicates). Very similar results were obtained between both devices, with values that differ by a maximum of 20%.

Table 2: Average peak areas, average peak heights and their RSD values (n=3) obtained by injecting a solution at 0.1 M of acetaminophen and acetanilide in EtOH or DCM respectively in the two-valve set-up or after its dilution (x1000 with ACN-water with 3 mM Am Ac) in the one-valve set-up. Valves from Rheodyne, 300 nL-loops in Peek.

Compound and initial solvent	m/z of detected ion	Solution (0.1 M) diluted by 1000 with ACN-water + 3 mM Am Ac and injected in the **One-valve set-up**		Solution (0.1 M) directly injected in the **Two-valves set-up**	
		Peak height (RSD, n=3)	Peak area (RSD, n=3)	Peak height (RSD, n=3)	Peak area (RSD, n=3)
Acetaminophen/EtOH	152.1	9.42 E6 (12.7%)	9.6 E7 (8.5%)	1.07 E7 (30.3%)	1.23 E8 (33.9%)
Acetanilide/DCM	136.1	1.59 E7 (18.4%)	1.96 E8 (20.4%)	1.45 E7 (17.7%)	1.96 E8 (16.9%)

The similarity of peak areas and peak heights obtained for compounds prepared in very different solvents, ethanol and DCM here, demonstrates the success of the dilution step done by the two-valves set-up, but high RSD values were observed that could certainly be improved. However, before that, it was interesting to first evaluate the possibility to replace manual injection of pure analytes diluted in a selected solvent by a direct coupling with the µR implementing the chemical reaction involving the same compounds.

4- First attempt to couple the microreactor to the two-valves set-up

Before connecting the µR directly to the two-valves set-up, the reaction media resulting from the synthesis of acetaminophen or acetanilide described in **Fig. S2** were manually injected with a syringe as previously done with the pure solutions of standards. **Fig. 3** allows the comparison of results obtained by a direct injection of a solution of 0.1 M acetanilide in DCM diluted x1000 before being injected at 0.1 mM in the one-valve set-up and a direct injection of the µR content recovered after the on-flow synthesis of acetanilide (expected at 0.1 M in the microreactor effluent) in the two-valves set-up. It is worthwhile to notice that the synthesis in the µR was achieved one month before the analysis of the resulting products.

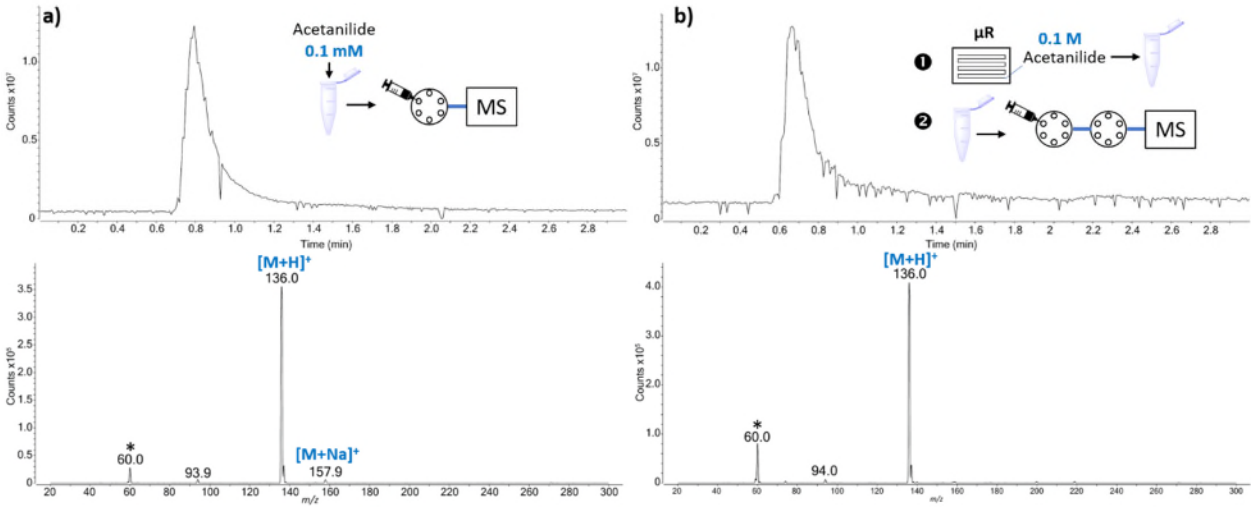

Fig. 3 Comparison of the EIPs (*m/z* 136.1) and average mass spectra obtained after the analysis (a) of a diluted (x1000 with ACN-water with 3 mM Am Ac) solution of acetanilide in DCM (0.1 mM) injected in the one-valve set-up and (b) of products resulting from the reaction of synthesis of acetanilide (expected at 0.1 M if a 100% conversion rate is obtained from aniline) achieved in the μR and injected in the two-valves set-up. Valves from Rheodyne, 300 nL-loop in Peek. The ion detected at *m/z* 60.0 in both mass spectra (asterisk) was detected in the blank sample and so is not proper to the samples.

The similarity of the EIPs corresponding to the signal specific to acetanilide ($[M+H]^+$ at *m/z* 136) and of the average mass spectra that evidences the presence of acetanilide first highlights the possibility to analyze the content of the microreactor that contains a complex medium including many reagents such as catalysts.

It is worthwhile to notice that the average mass spectra (**Fig. 3**) also evidenced a peak at *m/z* 94 of low intensity that could correspond to the pseudo-molecular ion of aniline, when acetanilide was analyzed with both systems. Similarly, a peak detected at m/z 110 of low intensity that could correspond to the pseudo-molecular ion of the 4-aminophenol was also observed when analyzing acetaminophen (data not shown). Actually, the presence of these ions would more result from the fragmentation of acetanilide or acetaminophen in the MS source. Indeed, the similarity of the signal ratios of *m/z* 110/*m/z* 152 and *m/z* 94/*m/z* 136 seems to confirm in-source fragmentation as they are similar using both one- and two-valves set-ups. It must be noticed that these ratio does not correspond to the respective proportion of the compared ions as the ionization efficiency is compound dependent. This hypothesis was checked by carrying out tests at the level of the ionization source by modifying the parameters which can generate fragmentation phenomena. Additionally, it was also evaluated by the injection of acetanilide and acetaminophen solutions in LC-MS (*i.e.* using a column) which led to a single chromatographic peak (results not shown), without revealing a second chromatographic peak specific to the presence of aniline or 4-aminophenol, respectively.

The next step consisted in comparing the one- and two-valves set-ups by manually injecting in both cases the effluent of the μR, implementing the two reactions leading to either acetaminophen

or acetanilide and done in two different solvents. Of course, in the one-valve set-up where the dilution by a factor 1000 of the sample is not included, it was performed before injected the µR effluent. The obtained results for the analysis of protonated acetaminophen ($[M+H]^+$ at *m/z* 152) and acetanilide ($[M+H]^+$ at *m/z* 136) produced in the µR and detected in MS are summarized in **Table 3** in the two columns entitled manual injection. Again, the similarity of the average peak heights and peak areas for either acetaminophen or acetanilide obtained with the one- and two-valves set-ups confirm its high potential for the analysis of the contents of the µR.

Table 3: Average peak areas and average peak heights (RSD values, n=3) obtained when injecting manually the products resulting from the synthesis in the µR of acetaminophen or acetanilide (reactants at 0.1 M in EtOH and DCM, respectively) in the two-valves set-up or after its dilution (x1000 with ACN-water and 3 mM Am Ac) in the one-valve set-up or when connecting directly on line the µR implementing the synthesis reactions of acetanilide (reactants at 0.1 M in DCM) or acetaminophen (reactants at 50 mM in EtOH) to the two-valves set-up.*n=2. Valve from Rheodyne, 300 nL-loop in Peek.

Reaction	**Detected ions**	**Manual injection of the diluted (x1000) µR content, One-valve set-up**		**Manual injection of the non-diluted µR content, Two-valves set-up**		**Direct coupling of the µR to the two-valves set-up**	
		Peak height (RSD, n=3)	Peak area (RSD, n=3)	Peak height (RSD, n=3)	Peak area (RSD, n=3)	Peak height (RSD, n=3)	Peak area (RSD, n=3)
Synthesis of acetaminophen	A: m/z 152	1.94 E6 (13.9%)	2.42 E7 (12.7%)	2.05 E6 (23.0%)	2.83 E7 (26.7%)	3.28 E6*	4.69 E7*
	B: m/z 110	8.03 E4 (6.0%)	1.45 E6 (10.2%)	9.32 E4 (17.6%)	1.81 E6 (18.0%)	3.54 E5*	3.79 E6*
	B/A ratio	4%	6%	5%	6%	11%	8%
Synthesis of acetanilide	A: m/z 136	1.02 E7 (2.9%)	1.17 E8 (7.5%)	1.17 E7 (17.6%)	1.49 E8 (20.1%)	1.11 E7 (54.4%)	1.37 E8 (49.2%)
	B: m/z 94	1.85 E5 (4.1%)	2.23 E6 (2.3%)	2.06 E5 (15.3%)	2.27 E6 (15.2%)	2.66 E5 (14.0%)	3.66 E5 (15.9%)
	B/A ratio	2%	2%	2%	2%	1%	2%

In offline conditions, even if higher, RSD values were obtained using the two-valves systems in comparison with the one-valve system (see Table 3), the intensities of MS signal are very similar. Therefore, manual injections of acetanilide or acetaminophen into the two-valves set-up (Fig. 4a) have been compared with injection of acetanilide or acetaminophen reactants in the µR (via two syringe pumps) directly coupled to the two-valves set-up (Fig. 4b). The aim is to measure peaks height and area to see whether or not similar values in both cases were obtained, values are reported in Table 3.. When using the µR, the duration of the injection of the effluent in loop 1 is 1 min and

the loop content is transferred to the loop 2 in 10 min, with MS analysis being performed in 2 min. Therefore, the two-valves set-up requires 13 min for the first analysis and then 10 min for each subsequent analysis as it can be started before the end of the previous one. The EIPs obtained when studying the synthesis reaction of acetanilide within the µR directly coupled to the two-valves set-up are reported in **Fig. 4b.** They can be compared to the EIPs obtained when injecting manually in the same two-valves set-up a standard solution of acetanilide (0.1 M in DCM) reported in **Figure 4a**. They are quite similar.

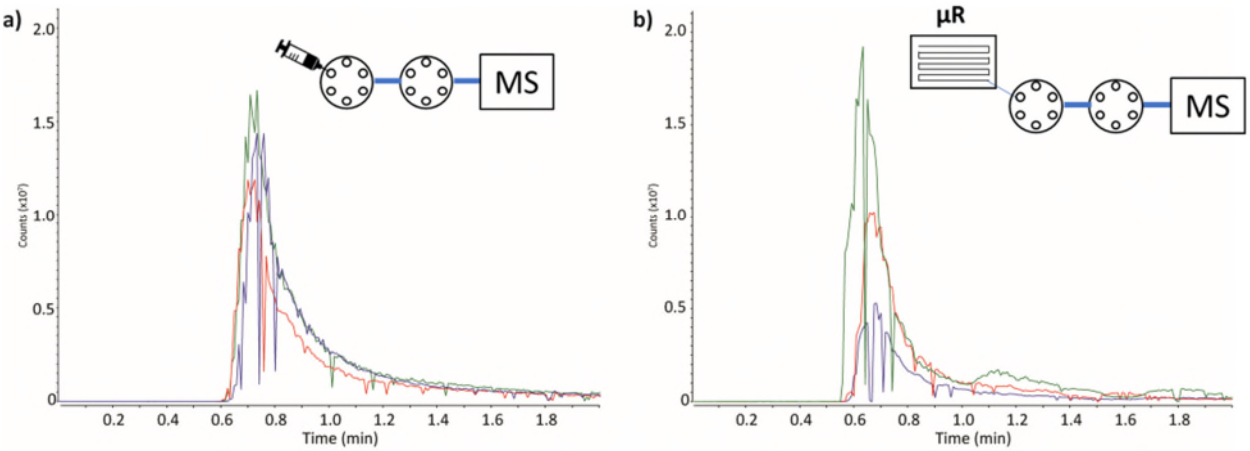

Fig. 4 EIPs (*m/z* 136.1) corresponding to (a) a manual injection of acetanilide 0.1 M in DCM in the two-valves set-up and (b) a direct coupling of the µR to the two-valves set-up to directly analyze the products of the synthesis reaction of acetanilide (reactants at 0.1 M in DCM).

For a more detailed study of the performance of this coupling, average peak heights and average peak areas obtained when studying both reactions are reported in **Table 3** (last column). Note that the analyzed concentration of acetaminophen was 0.05 M instead of the previous studies done at 0.1 M. With results illustrated in **Fig. 4** and reported in **Table 3**, it can be concluded that the on-line coupling of the µR with the two-valves set-up was successfully achieved, as it allows the detection of the reaction products (acetaminophen or acetanilide) with similar intensities as for a manual injection, taking into consideration the initial reactant concentrations. However, the lack of repeatability of the results (high RSD values in **Table 3**) and the long analysis time (13 min) were the trigger to implement some improvements trying to reduce some void volumes of the system.

5- Improvements of two-valves set-up in terms of analytical time and repeatability

A reduction of the analytical time has been investigated by reducing the length of the capillaries between the two valves (6 cm to 4cm) and the volume of the second loop (300 nL to 150nL), since the constraint linked to the back-pressure exists only for the first valve connected to the µR (**Figure S4a**). After checking that the reduction of the sample loop still allowed the good detectability of acetaminophen in MS, a new optimization of the transfer time between the two loops was

performed and assessed to be more than 5 min (**Fig. S4b**). The transfer time was then fixed at 6 min and, in these conditions, the first analysis required 9 min and then only 6 min are now required for subsequent analyses. The content of the microreactor can be then analyzed every 6 min.

Once the transfer time optimized, it was interesting to optimize the scan time of the quadrupole mass analyzer. Indeed, a non-adapted scan time could be the source of instability. While in mass spectrometry long scan times gives well-defined peaks, in elution process as in chromatography this is not the case. Therefore, a compromise must be made between peak width and MS peak resolution. For that, different scan times were tested and the average area and area RSD values of the MS peak of acetaminophen were evaluated for each of it. No tendency was observed apart from an important instability of the signal when measuring the peak area or peak heights of the EIP of acetaminophen (**Fig. S5**). As the set-up includes two valves, a T-piece for dilution, a split, and different connections tubes, the instability could come from numerous parts of this system. Indeed, while the two-valves set-up can be subjected to bad connections or dead volumes (especially when nanofluidic is used), many problems could arise also from the mass spectrometer (bad ionization efficiency, ionization source and analyzer parameters not optimized, etc…).

To identify the source of this instability, the two-valves set-up was hyphenated to a simple ultraviolet (UV) detector instead of the mass spectrometer (see **Fig. S6a**). Before evaluating this new system, the UV detector was connected just behind the first loop and acetaminophen (50 µM in ACN-water (1:1) + 3 mM Am. Ac.) was manually injected and detected at 245 nm showing an average peak height of 93.3 mAU with a RSD value of 1.96% (n=3). In return, when injecting the same solution with the two-valves set-up connected to UV (**Fig. S6a**) and repeating the injection with different transfer times, no linearity of the signal was obtained, indicating that the optimal condition was not reached (**Fig. S7a**). While connecting the UV detector instead of the waste in the loop 2, the UV signal was growing up to 30 min of transfer (**Fig. S7b**), thus confirming that the transfer time was much longer than expected.

To reduce this long transfer time and to try to elucidate the source of instability of the MS signal, valves and loops initially tested were changed. A valve from another supplier (VICI-Valco, C82H-6676 valve instead of 7010-Rheodyne valve) was used. This 6-port 2-position valve is composed of a microbore injector with port size of 150 µm that is certainly lower than that of the Rheodyne valve for which no value is provided. If it differs by a smaller port-to-port volume, the system would have a lower void volume and so the transfer time would be reduced. A new sample loop was made using a fused-silica capillary (internal diameter 51.15 µm, length 7.3 cm) instead of peek tubing to ensure a better and tight connection. A low volume of the first loop was kept at 150 nL (see set-up in **Fig. S6b)**. The transfer volume was then again studied (see **Fig. S8**). The highest intensity of the peak was obtained with a transfer time of only 1.25 min, leading to a peak height of 92.8 mAU, *i.e.* a value similar to the one previously obtained when injecting manually the same acetaminophen solution in the single valve equipped with a 150 nL loop directly connected to the UV detector. This good result confirmed that the too high transfer time previously obtained (> 30 min) was due to the use of a Rheodyne valve with a larger void volume.

To respect the backpressure limitation coming from the µR, the fused-silica based-loop of 150 nL placed on the valve 1 was replaced by a new fused-silica loop of 300 nL, keeping the 150 nL loop on valve 2. Thus, if we compare the final two-valves set-up to the initial one (**Fig. 1c**), the optimization led to the replacement of the 300 nL loop by a 150 nL loop on valve 2, a Valco valve was used as valve 1 to reduce the void volumes and the loops are now made of silica and not of peek. The transfer time was then again studied with the set-up coupled to UV and then confirmed using the MS detector. **Fig. 5** illustrates the effect of the transfer time on the peak height of acetaminophen using the optimized two-valves setup coupled to either UV (**Fig. 5a**) or MS (**Fig. 5b**) detector.

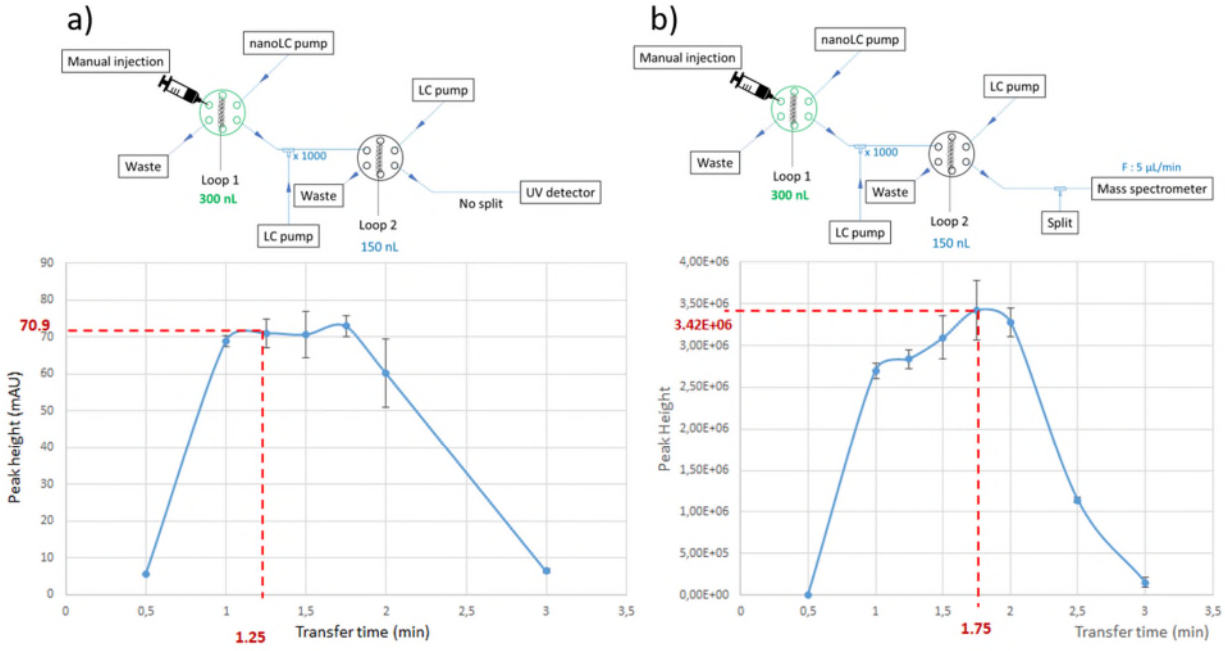

Fig. 5 Study of the transfer time with the optimized two-valves set-up coupled to either UV (a) or MS (b) detection. Manual injection of acetaminophen in EtOH at 50 mM (n=3). Valve 1 from Valco and valve 2 from Rheodyne, loops of 300 and 150 nL made of fused-silica.

In both cases, it can be concluded that the optimal transfer time was obtained between 1 and 1.75 min. Moreover, the stability problem was fixed by the modifications of the set-up as the RSD values are now low (0.78% for n=3). These instabilities came from the dead volumes inherent to the type of valve and loop used. Finally, the transfer time was fixed at 1.75 min which allowed repeatable measurements in MS and a total of 4 min for each analysis with this modified set-up.
These satisfactory results obtained in terms of repeatability allowed the study of the effect of MS scan times on the MS signal. Different scan times (from 200 till 1000 ms) were tested for the analysis of acetaminophen with the optimized two-valves set-up and a stable and repeatable MS signal was obtained starting from 400 ms. Therefore, the scan time was fixed to 500 ms to insure the signal stability.

6- Evaluation of the optimized two-valves set-up with non-MS compatible solvents

The optimized two-valves set-up was further evaluated by studying the linearity of the response when injecting acetaminophen at different levels of concentration in ethanol (3.1-100 mM). In addition, to evaluate the capacity of the set-up to analyze compounds in a non-MS compatible solvent, acetaminophen was also injected in DMSO at different concentrations (12.5-100 mM). The intensity and area of the resulting MS signals are reported in **Fig. S9**. In both cases, the signal is repeatable and proportional to the concentration assessing the linearity of the system even for the worst solvent, DMSO here, even if the intensity of the signal of acetaminophen is lower in DMSO than in EtOH (loss of about 30%). It should be noticed that no optimization of the parameters and conditions of ionization of the acetaminophen solubilized in DMSO was performed. The observed loss in intensity but also the large errors bars observed in Fig. S9 could be explained by this lack of optimization associated to an ion suppression effect probably caused by the simultaneous ionization of DMSO.

To complete this study, the universality of the optimized two-valves set-up was evaluated by applying it to the detection of various molecules dissolved in various solvents previously presented in **Table S2**. Each molecule was prepared in ethanol, acetone, DMF and DMSO at 50 mM when its solubility was adapted or its availability was sufficient. The corresponding average peak area (n=3) was reported as a function of the solvent in **Table 4**. The highest signal intensities were mainly obtained when the molecules were diluted with ethanol whereas the other solvents induced a decrease in the average area values. Therefore, the solvent used significantly affects the signal intensity of all the tested compounds. In general, DMSO and DMF, to a lesser extent, caused ion suppression of the tested molecules. Regardless of this ion suppression, the system proved its ability to detect those molecules even when non-MS compatible solvents are used for molecule solubilization due to the efficiency of the dilution procedure with a highly MS compatible acidic hydro-organic mixture. Therefore, the developed system proved its universality: it can be applied to various molecules associated with different solvents challenging in MS, as long as the molecules of interest are soluble in them.

Table 4: Average peak areas (RSD values, n=3) of each compound prepared in different solvents and analyzed by the optimized two-valves set-up with the MS detector. For a given compound, the highest peak area value is reported in bold and the percentages in the column correspond to the losses in signal intensities compared to the highest signal value. X: molecule not soluble in the corresponding solvent.

Compound	EtOH		Acetone		DMF		DMSO	
	Av. peak area	Loss	Av. peak area	Loss	Av. peak area	Loss	Av. peak area	Loss
Rotigotine	**7.3** (2.5%)	-	7.1 (7.0%)	3%	5.8 (9.9%)	20%	6.0 (11.9%)	18%
Valsartan	**1.3** (2.0%)	-	1.1 (2.0%)	15%	0.87 (2.0%)	31%	0.5 (14.5%)	61%

Rivastigmine	8.5 (15.2%)	36%	**13.2** (6.9%)	-	10.4 (1.2%)	21%	9.4 (14.6%)	29%
Lidocaine	**34.8** (8.0%)	-	33.0 (11.8%)	5%	20.9 (9.0%)	40%	21.2 (9.6%)	39%
Trimethobenz.	X		X		13.8 (5.3%)	7%	**14.9 (**2.5%)	-
Levofloxacin	X		**4.14** (13.5%)	-	1.9 (15.6%)	53%	2.7 (25.0%)	35%
Sitagliptine	X		X		2.3 (10.0%)	4%	**2.45** (9.4%)	-
Acetaminophen	**5.1** (4.9%)	-	4.9 (1.8%)	4%	4.2 (3.8)	16%	2.1 (5.2%)	57%

7- Coupling of the optimized two-valves set-up to the µR to monitor a chemical reaction

The optimized two-valves set-up was then applied to the analysis of the products of the reaction of amidation carried out in DCM that was selected as a model reaction. Indeed, this solvent is often used for chemical reaction. It is also weakly soluble in water (17.5 g/L) compared to methanol (fully soluble) and is not suitable for electrospray ionization as it is too volatile. The selected reaction, described in **Fig. S10**, involves two reactants (named R1 and R2) and two coupling reagents (EDC and HOBt) that lead to the formation of two products (P1 and P2).

This reaction was achieved by a simple shaking in a vial but also in the µR. Firstly, the two resulting samples were manually injected in the two-valves set-up. The MS spectra obtained after ionization in positive ion mode of each samples are reported in **Fig. S11**. First, the similarity of the two MS spectra resulting from a similar reaction without any purification and injected in the developed set-up confirms the reliability of the system to analyze compounds initially present in a not fully compatible MS solvent. In addition, two ions, *m/z* 174 and *m/z* 269, diagnostic of the pseudo-molecular ions $[M+H]^+$, of the two expected products, P1 and P2, are present in both spectra. Note that the ion detected at *m/z* 269 has an isotopic profile characteristic of the presence of a chloride atom supporting the fact that this ion is the $[M+H]^+$ of P1. The RSD values of the intensities of P1 and P2 obtained for the analysis in triplicate of each sample are lower than 18.2%, confirming reliability. One of the coupling reagents, HOBt ($[M+H]^+$ at *m/z* 136), was detected in both samples while no ion diagnostic of the presence of the second coupling reagent (EDC) or of the two reactants (R1 and R2) was detected in any of the samples. On the contrary, two unknown ions (*m/z* 129 and *m/z* 222) were detected in both samples. To try to identify these products, CID experiments were performed using the two-valves set-up with the mass spectrometer operating in MS/MS mode. The corresponding profiles and potential structures are represented in **Figure S12**. Unfortunately, the ion *m/z* 222 is so poorly intense that its dissociation could not be done. To confirm the proposed structures, the elemental composition of the precursor ions and their fragments were validated using a high-resolution mass spectrometer (QTOF) by direct infusion of the diluted µR effluent (not shown). Concerning *m/z* 129, its high intensity on the MS/MS spectrum of P2 (**Figure S12a**) at such collision energy let us think to an in-source dissociation of P2.

These promising results made it possible to consider the direct on-line coupling of the µR implementing the same amination reaction with the optimized two-valves set-up. In this case, the microsystem effluent was monitored every 4 min. To illustrate the obtained results, **Fig. 6** presents the MS spectrum of an EIP obtained for one of the three consecutive analysis of the µR effluent coupled on-line to the two-valves set-up. This spectrum shows the presence of P1 and P2 with RSD values of peak intensity lower than 16%, thus highlighting the stability of this coupling. This spectrum also evidences the presence of EDC and R2 that were not detected using manual injection of the effluent. One hypothesis can be a lack of stability of these reactants that cannot be detected if the solution is not quickly analyzed after the synthesis.

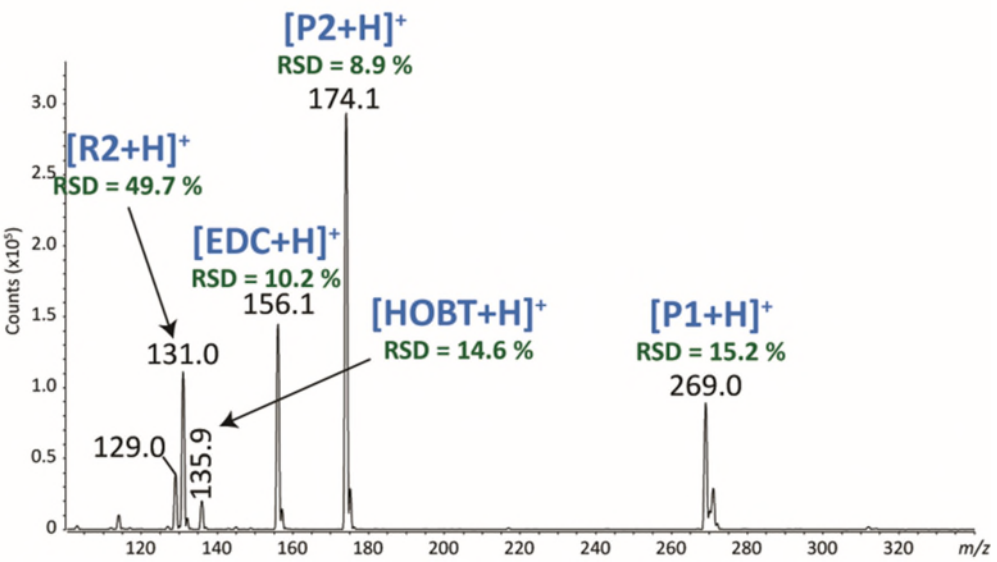

Fig. 6 Average mass spectrum resulting from an EIP obtained for the analysis of the µR effluent coupled on-line to the two-valves set-up. RSD values correspond to results from three consecutive analyses obtained with the same device.

Conclusions

The main goal of this research was achieved by the successful development of a universal two-valves set-up allowing to monitor on-line the effluent of a microreactor where the synthesis of various molecules in different solvents can take place. After fixing the detection conditions using acetaminophen, the set-up was developed using this acid molecule and a basic one, acetanilide, prepared in two solvents, one MS and one non-MS compatible, ethanol and dichloromethane, respectively. A dilution step with a high rate of 1000 and with a highly MS compatible acidic hydro-organic mixture and the use of two valves with adapted void volume and connected loops allowed to detect up to 11 molecules belonging to a wide range of polarity and of acido-basic properties in four common but more or less MS compatible solvents including DMSO. The developed two-valves set-up has proven to provide a repeatable and linear response for the

detection of the studied compounds in MS. This proof of concept was followed by its application to the MS monitoring of a chemical reaction achieved in a microreactor. The ability of the set-up to detect the expected products in addition to other byproducts that could be created along the synthesis process was illustrated. Finally, a successful on-line coupling of the microreactor to the two-valves set-up was achieved and has shown its capacity to monitor the content of the effluent of a microreactor every 4 min with a good repeatability as RSD values of MS signals were lower than 17%. Of note the developed two-valves set-up could be linked to any atmospheric pressure ionization source of any mass spectrometer configurations giving the opportunity to take advantage of all mass spectrometer technologies and their related analytical performances. In the future, the addition of an LC column in the set-up for the separation of compounds present in the microreactor effluent could be considered. This could facilitate the identification of the compounds and then make it easier to distinguish between the ions related to the compounds actually present in the effluent and the ions produced in the MS source.

Credit author statement

Christophe Chendo, Amira Al Matari, Thomas Bouvarel, Maël Arveiler: Conceptualization, Methodology, Investigation ; Christophe Chendo, Amira Al Matari, : Writing the original draft. Christophe Chendo, Nathalie Delaunay, Valérie Pichon: Visualization, Methodology, Writing – review & editing. Valerie Pichon, Olivier Venier, Michael Tatoulian : Project administration, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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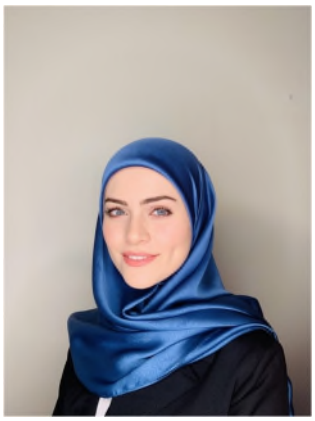

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Development of set-ups for real-time analysis of the effluent of a microreactor by mass spectrometry

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SUPPLEMENTARY MATERIAL

Table S1: Bibliographic table summarizing studies related to the monitoring of chemical reactions achieved in reactor, mainly in µR, by MS.

Aim of the study	Synthesis solvent	$C_{reagent}$	Set-up	Dilution solvent, Flow rate	Split before MS	Flow rate to MS	Analysis frequency	Goal	Ref.
Study of radical cation chain reactions in solution (Diels-Alder) by ESI-MS	DCM	0.1-1 mM	Two capillaries connected with a T-piece	-	-	2.5-100 µL/min for reaction time of 0.7 or 0.9 to 28 s	Continuous flow	QL	[7]
Detection of transient radical cations in electron transfer-initiated Diels-Alder reactions by ESI-MS		0.1-5 mM							[1]
Monitoring of Brookhart polymerization by ESI-MS	Toluene	n.a.							[9]
Study of the kinetics of isocyanate derivatization reactions by Chip-based on-line nanoESI-MS	ACN	1-5 µM	Direct flow from chip-based µR			20-100 nL/min		SQ	[8]
Study of homogeneously catalyzed Ziegler-Natta	Toluene (5-50 µL/min):	10-20 µM	Chip-based µR + T-piece for dilution	ACN, -	-	> 5-50 µL/min for reaction time of 0.3-1.7 s	Continuous flow	QL	[10]

polymerization of ethene by ESI-MS									
Monitoring of RAFT polymerization by ESI-MS	Butyl acetate	0.7-15 mM	Chip-based µR (4 µl/min) + 2 T-pieces to dilute and split	x500 THF-MeOH, 6:4, 2 ml/min	1/400	5 µl/min	Continuous flow - > 3.13 min (dead time)	SQ	[11]
On-line monitoring of a Diels-Alder reaction by ESI-MS	ACN (0.1 ml/min)	200-240 mM	Reactor coil + loop (5 µl) on a switching valve + 2 T-pieces to dilute and split	x10 ACN-H_2O (1:1) + 0.1% FA, 0.9 ml/min	1/2000	0.5 µL/min	1 sample every 4-6 min	SQ	[12]
Monitoring of Morita-Baylis-Hillman reaction using a droplet-based µchip integrating a nanoESI emitter	Water	65-130 mM	On-chip digital µR (droplets of 1.1 µL) + 2 successive dilution steps by merging droplets	x5 H_2O, x2 MeOH + 0.1% FA	-	dynamic and static migration of droplets	> 10 min	QL	[14]
Monitoring of Hofmann rearrangement using a portable MS	2-MeTHF	40-60 mM	µR connected to MRA to aliquot/dilute the reaction media (100 µl) + split (not described)	x1000 ACN-H_2O (2:8) + 0.1% FA + IS	1/999	1 µL/min	8 min	SQ	[3]
Monitoring of amidation reaction using APCI-MS	MeOH	1.46-5.77 M	3 ml-Reactor coil + loop (60 nL) connected on a switching valve + switching valve/T-piece to split	H_2O-ACN 1:1 + 0.1% FA, 300 µl/min	1/2.5	120 µL/min	60 s	QT	[15]

Process monitoring of nucleophilic substitution reaction with NMR and MS	MeOH	2-60 µM	MRA to aliquot/dilute a pumped **batch reaction** media + split (not described)	x 4000 to 6666, H_2O-ACN 4:6 + 0.1% FA, 2 ml/min	1/4.7	400 µl/min	10 min	QL	[16]
Monitoring of oxidation reaction profiling by MS	Toluene	200 mM	**Flow injection** (0.1 ml/min) in MRA to aliquot/dilute the pumped batch reaction media + split (not described)	x 100-3300, ACN + 0.1% FA, 1 ml/min	1/1000	1 µL/min	> 3 min	QT	[17]

APCI: Atmospheric pressure chemical ionization; FA: formic acid; IS: internal standard. MRA: mass rate attenuator. QL: Qualitative study; SQ: semi-quantitative study; QT: quantitative study.

Table S2: Some physico-chemical parameters of the selected solvents. (*: recommended concentration of DMSO for the analysis of low molecular weight molecules in ESI-MS)

Solvent	Polarity index	Density	Viscosity at 20°C (mPa s)	Solubility in H_2O at 20°C	Miscibility in ACN-H_2O (50/50)	MS recommended concentration*
Toluene	2.4	0.87	0.59	0.5 g/L	1% (v/v)	-
Dichloromethane	3.1	1.33	0.44	13-20 g/L	2% (v/v)	-
Ethyl Acetate	4.4	0.90	0.45	83 g/L	10% (v/v)	-
Dioxane	4.8	1.03	1.54	Any proportion	> 50% (v/v)	-
Methanol	5.1	0.79	0.60	Any proportion	> 50% (v/v)	-
Acetonitrile	5.8	0.79	0.37	Any proportion	-	-
Dimethyl formamide	6.4	0.94	0.92	Any proportion	> 50% (v/v)	0.1% (v/v)
Dimethyl sulfoxide	7.2	1.10	2.00	1000 g/L	> 50% (v/v)	0.1% (v/v)
Water	9.0	1.00	1.00	-	-	-

Table S3: Effect of the dimension of the loop (made of a capillary) on the back-pressure when percolating water at 50 or 500 µl/min, each capillary used to design a loop of a given volume having a length of 10 cm (outer diameter of 1/16 inch) to be connected to the valve.

Loop volume (nL)	Internal diameter of the capillary (µm)	Back-pressure at 50 µL/min (bar)	Back-pressure at 500 µL/min (bar)
49	25	65	> 200
330	65	1.1	10.7
785	100	0.2	1.6
1227	125	0.1	0.9
1767	150	< 0.1	< 0.1

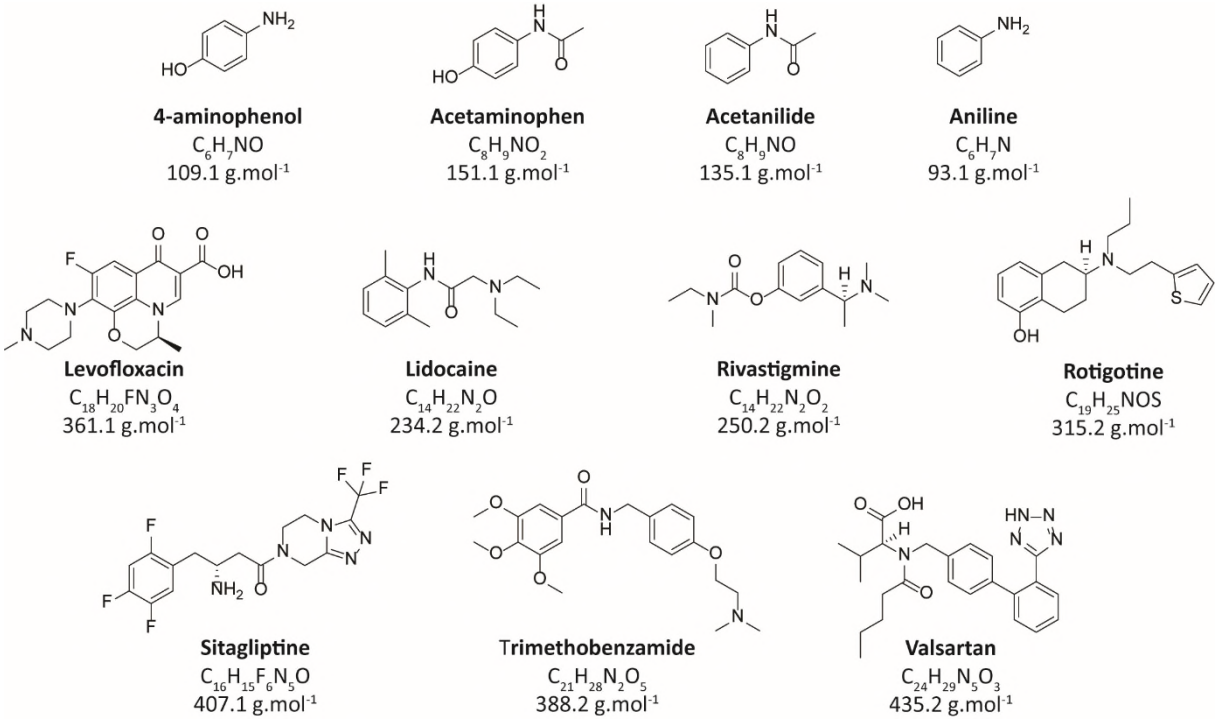


Figure S1: Structure and molecular weight of the model molecules

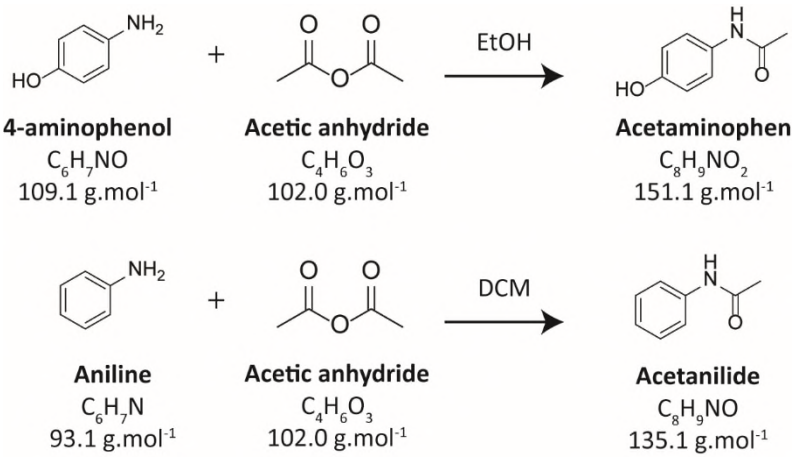


Figure S2: Synthesis of acetaminophen (paracetamol) resulting from the reaction of 4-aminophenol with acetic anhydride in ethanol (top) or synthesis of acetanilide from aniline and acetic anhydride in dichloromethane (bottom).

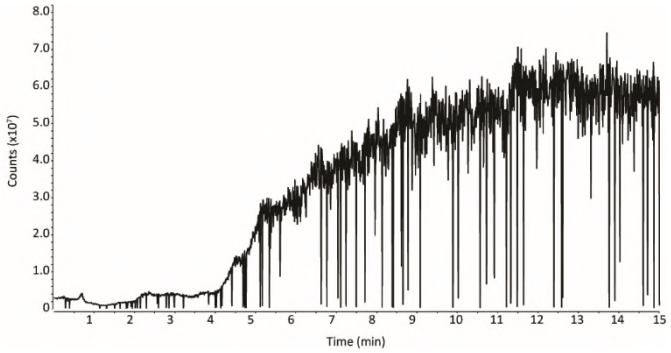


Figure S3: Extracted ion profiles (EIPs) of acetaminophen, (*m/z* 152.1) injected at 0.1 M in ethanol in the one-valve set-up described in Figure 1b. Each injection was done in triplicate. Valves from Rheodyne, 300 nL-loops in Peek.

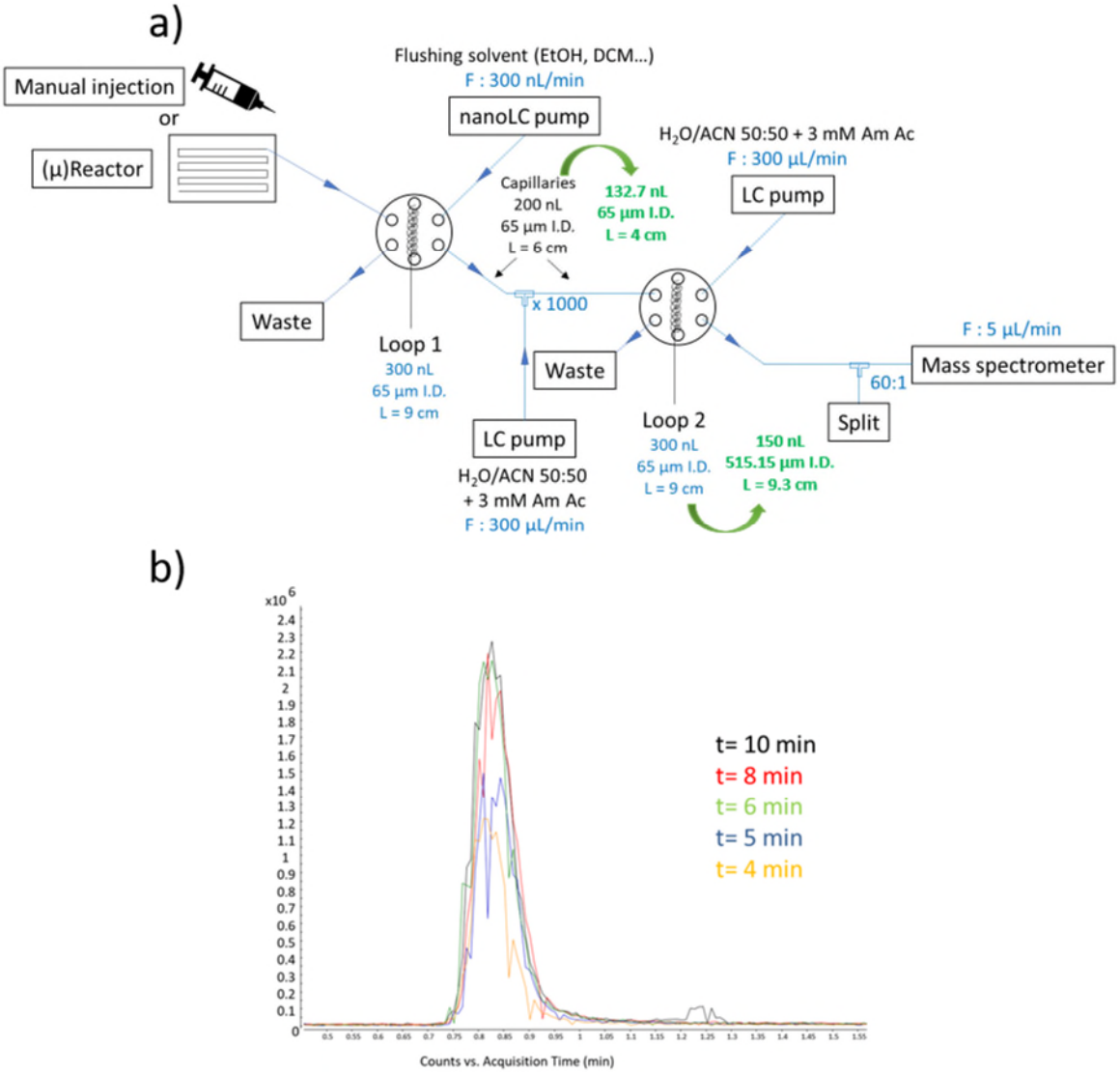


Figure S4: Scheme presenting the changes (in green) operated in the two-valve set-up to reduce the analysis time (a) and (b) EIPs of paracetamol (*m/z* 152) obtained to determine the optimized transfer time with this two-valve set-up.

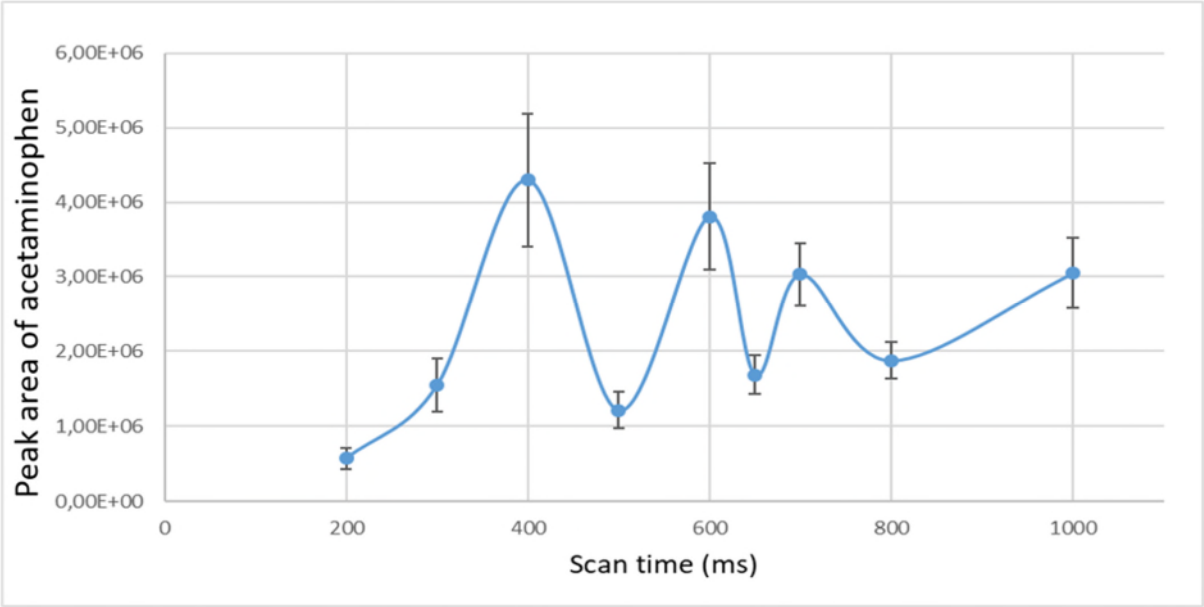


Figure S5: Evolution of the peak area of acetaminophen as a function of the scan time of the quadrupole mass analyzer. Experiments were performed in triplicate after a manual infusion of the acetaminophen solution in the two-valve set-up.

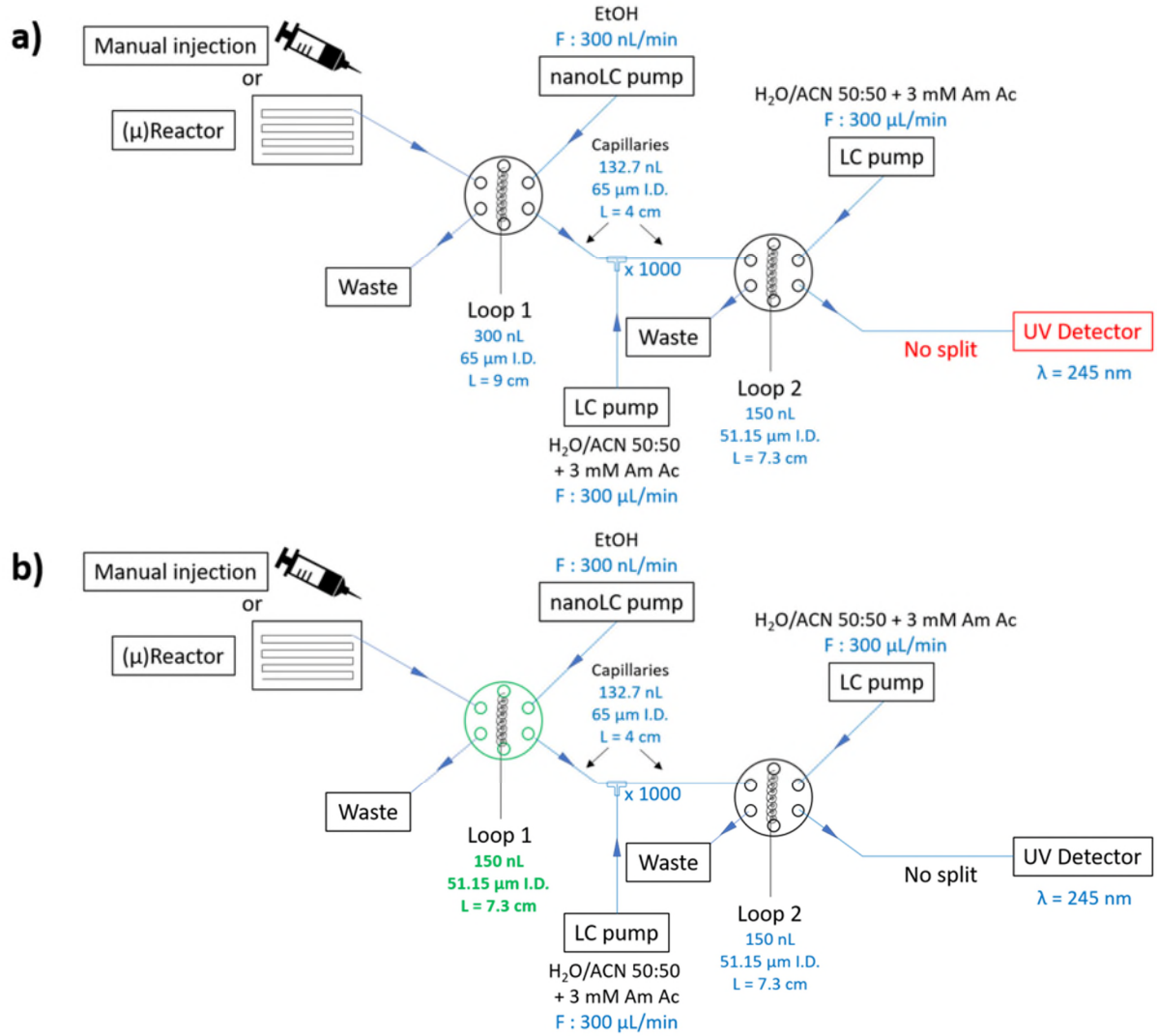


Figure S6: Scheme of the two-valves set-up connected to UV and made of valves from Rheodyne and loops in Peek (a) and new two-valves set-up equipped with a home-made loop 1 of 150 nL prepared with a fused-silica capillary and a Valco switching valve 1 (b).

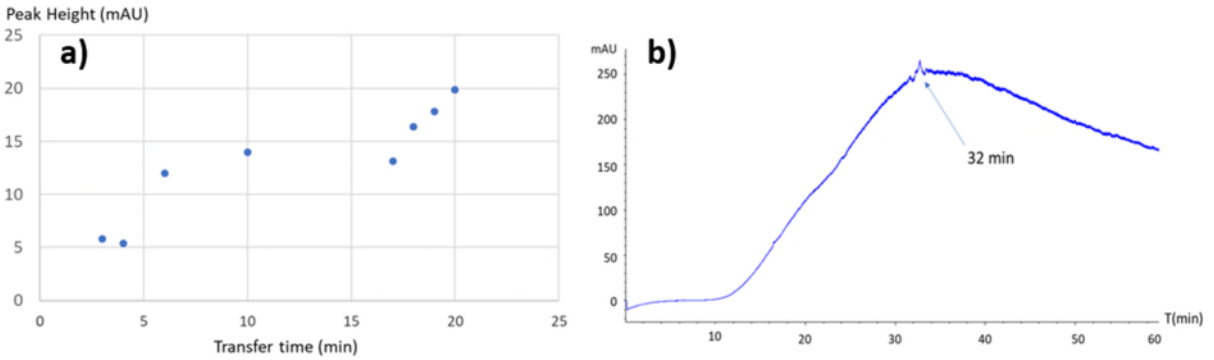


Figure S7: Effect of the transfer time on the peak height of acetaminophen (50 mM in EtOH) analyzed using the set up described in Figure S5a (a) and evolution of the UV signal of acetaminophen when connecting the UV detector between the dilution T-piece and the valve 2 (a). Valves from Rheodyne, loops in Peek.

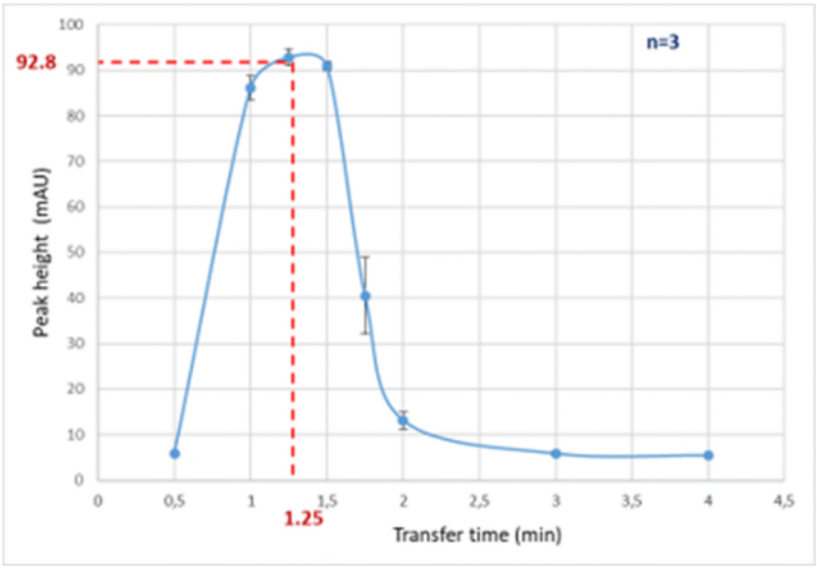


Figure S8: Effect of different transfer times on the peak height of acetaminophen (50 mM) analyzed using the two-valves set-up connected to a UV detector (245 nm) using the set-up depicted in Figure 5a (including Valve 1 from Valco connected to a loop (150 nL) in fused silica capillary).

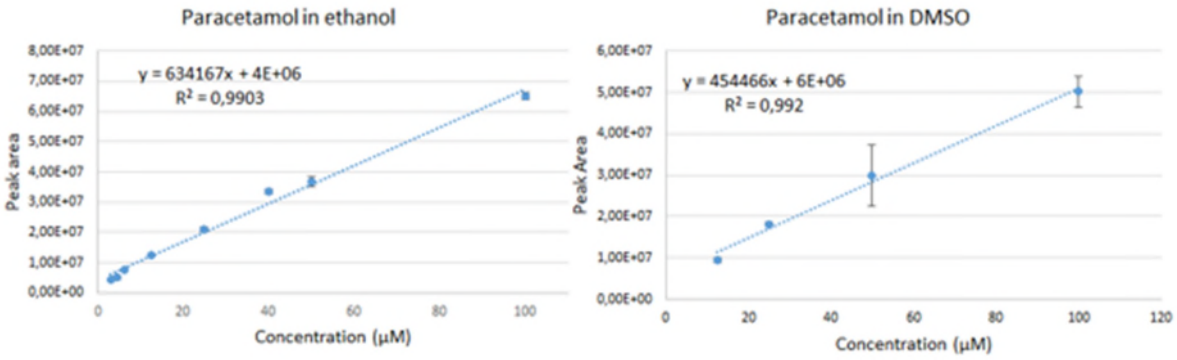
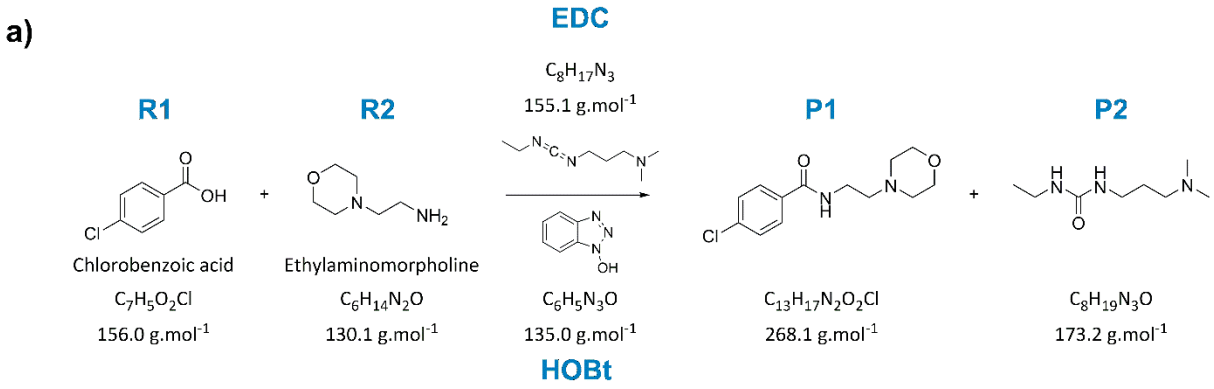


Figure S9: Injection of acetaminophen (paracetamol) at different concentrations in EtOH or DMSO in the optimized two-valves set-up (described in Figure 5b). Each injection was done in triplicate.



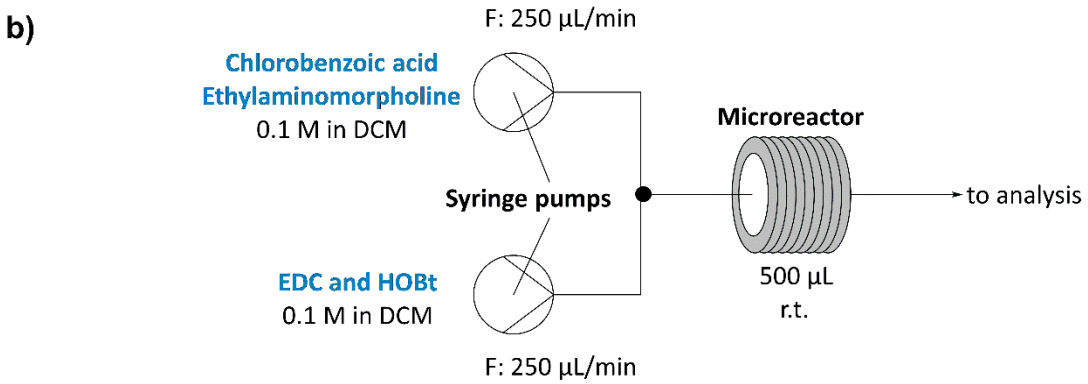


Figure S10: a) Amination reaction scheme presenting the reactants, the coupling reagents, and the expected synthesized products with their elemental composition and their monoisotopic mass. b) Schematic representation of the synthesis performed in the microreactor, reactants R1, R2 and EDC, HOBt are introduced in the microreactor by 2 syringe pumps operating at a flow rate of 250 µL.min^{-1}.

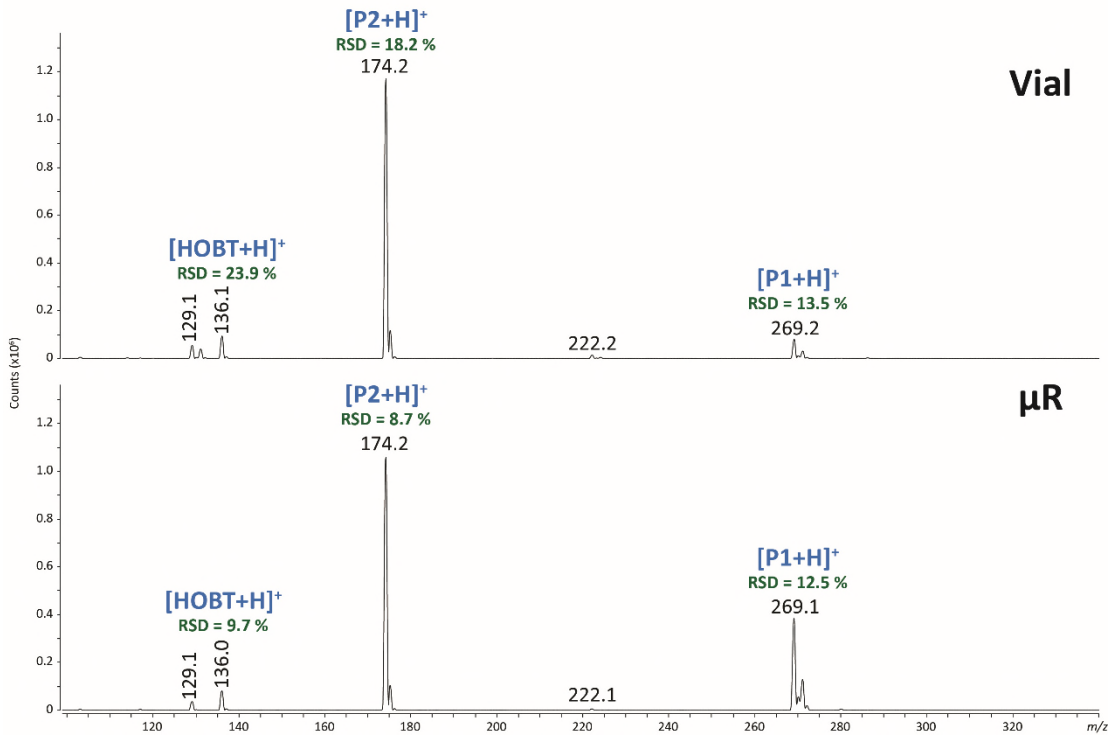


Figure S11: Mass spectra of the samples resulting from the amination reaction achieved by a simple shaking in a vial or in the µR and manually injected in the optimized two-valves set-up hyphenated to MS with an electrospray ionization in positive ion mode (RSD values of MS peaks height obtained for the analysis in triplicate).

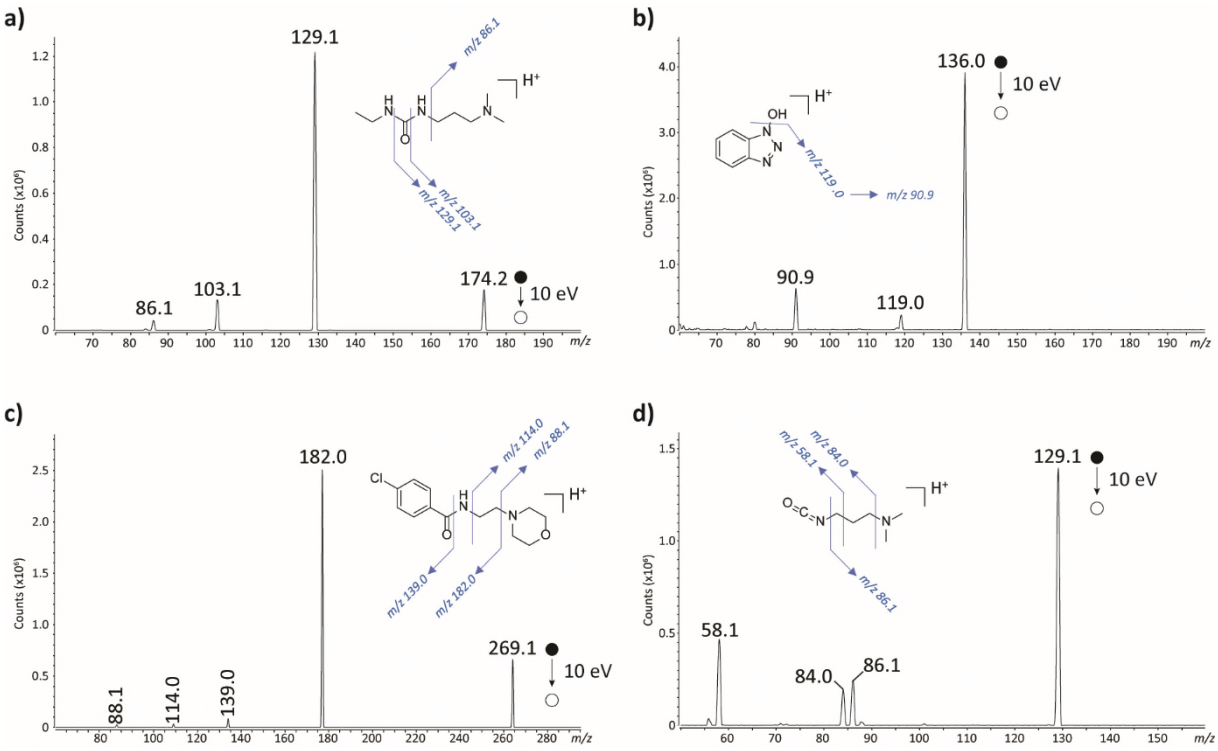


Figure S12: Collision induced dissociation spectra at 10 eV collision energy and dissociation scheme of the protonated molecules obtained after MS/MS analysis of the amination reaction using the two-valves set-up.