Ultrasonication-Assisted Spray Ionization-based Micro-reactors for On-line Monitoring of Fast Chemical Reactions by Mass Spectrometry

Te-Yu Chen, Min-Li Wu, and Yu-Chie Chen*

Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan

*Corresponding author
E-mail: yuchie@mail.nctu.edu.tw
Fax: +886-3-5723764
Phone: +886-3-5131527

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Abstract

Microfluidics can be used to handle relatively small volumes of samples and to conduct reactions in microliter-sized volumes. Electrospray ionization (ESI) can couple microfluidics with mass spectrometry (MS) to monitor chemical reactions on-line. However, fabricating microfluidic chips is time-consuming. We herein propose the use of a micro-reactor that is sustained by two capillaries and an ultrasonicator. The inlets of the capillaries were individually immersed to two different sample vials that were subjected to the ultrasonicator. The tapered outlets of the two capillaries were placed cross with an angle of ~60° close to the inlet of the mass spectrometer to fuse the eluents. On the basis of capillary action and ultrasonication, the samples from the two capillaries can be continuously directed to the capillary outlets and fuse simultaneously to generate gas phase ions for MS analysis through ultrasonication-assisted spray ionization (UASI). Any electric contact applied on the capillaries is not required. Nevertheless, UASI spray derived from the eluents can readily occur in front of the mass spectrometer. That is, a micro-reactor was created from the fusing of the eluent containing different reactants from these two UASI capillaries, allowing reactions to be conducted *in situ*. The solvent in the fused droplets was evaporated quickly, and the product ions could be immediately observed by MS because of the extreme rise in the concentration of the reactants. For proof of concept, pyrazole synthesis reaction and cortisone derivatization by Girard T reagent were selected as the model reactions. The results demonstrated the feasibility of using UASI-based micro-reactor for on-line MS analysis to detect reaction intermediates and products.

Introduction

Microfluidics and lab-on-chip technologies are popular because of their small sample consumption and their suitability for conducting fast chemical reactions.[1–4] Nevertheless, fabricating microfluidic devices is time-consuming. Moreover, specific equipment and clean rooms for device fabrication are usually required.[5,6] Microfluidic systems generally involve aqueous samples with low flow rates. Therefore, electrospray ionization mass spectrometry (ESI–MS) [7–9] that introduce liquid samples with a similar injection flow rate to that used in the microfluidic device is a suitable detection tool for on-line interfacing with microfluidic devices. [10–17] Coupling microfluidic chips with ESI–MS has been studied since the late 1990s.[10,11] External voltage can be applied to the sample reservoir to assist the electrospray formation at the edge of the microfluidic chip. Moreover, generating a stable electrospray is possible from the end of the microfluidic channels by inserting fused-silica capillaries as the ESI emitters.[17]

Fast chemical reactions can be completed within few seconds. Thus, fused-droplets that mixed droplets containing different reactants have been employed in conducting fast chemical reactions in front of the mass spectrometer.[18-21] These ESI derived MS approaches including fused droplet ESI,[18] extractive ESI,[19,20] and microdroplet MS[21] have been successfully used to monitor fast reactions on-line with MS in real time. Nevertheless, these approaches require external accessories such as high voltage power supply for generating electrospray. Ultrasonication-assisted spray ionization (UASI)[22–25] is also an ionization technique derived from ESI. The setup of the UASI includes a sample vial, a tapered capillary, and an ultrasonicator. The capillary directs the sample solution from the inlet in the sample vial that is subjected into the ultrasonicator to the tapered outlet. The tapered outlet works as an ESI emitter. The electrospray in UASI can be generated and facilitated by ultrasonication and the high voltage applied on the inlet of the mass spectrometer. The setup is unique

because applying direct electric contact to the capillary is not needed, making the operation straightforward and simple. The electrospray can be readily generated when placing the capillary outlet close (~3 mm) to the mass spectrometer inlet applied with a high electric voltage. Capillaries have similar channel sizes, as used in microfluidics. Thus, we can use two UASI capillaries instead of microfluidic channels to deliver reactants to an intersection and create a micro-reactor-like space on the air to conduct chemical reactions. Therefore, labor-intensive fabrication steps for making microfluidic channels can be eliminated. Furthermore, the UASI–MS approach demonstrated that the eluent from the capillary outlet, which is placed close to the inlet of the mass spectrometer applied with a high electric voltage, can be readily ionized. No direct electric contact applied on the capillary and its eluent is beneficial because possible effects derived from direct electric contact made on the reaction is eliminated. The generated ions can be instantly detected by the mass spectrometer. That is, the proposed setup can conduct micro-reactions on-line, and the resultant reaction species can be simultaneously monitored in real time by MS. As the solvent quickly evaporates, the concentration of the reactants rises quickly in the fused droplets, leading to the fast formation of products. Thus, production ions can be easily observed in the resultant UASI mass spectra.

Chemical reactions, such as pyrazole synthesis reactions[26] and Schiff base reactions[27] that can proceed rapidly and can be simultaneously detected by UASI-MS were selected as the model reactions. Pyrazole and its derivatives have been used as antiviral[28], anti-tumor[29], and antidepressant[30] medicines. Usually, several tens of minutes are required to complete a pyrazole reaction.[31] The reaction of phenylhydrazine and acetylacetone was one of typical pyrazole reactions, which can be conducted by using strong acid as the catalyst. ~1 h is usually required to complete the reaction.[31] Nevertheless, our approach can be used to observe the product ions during fusing of reactant droplets. In addition, Girard T reagent has been used as the reagent to form derivatives of aldehyde- and ketone-containing

chemicals.[32-34] The derivatized products generally can be easily observed in mass spectra because Girard T reagent contains a pre-charged functional group, i.e. (carboxymethyl)trimethylammonium, leading to improved ionization efficiency. Cortisone is a hormone that can be used to indicate the health of human beings.[35,36] It can be used as a medicine for treating diseases such as keloids.[37] Cortisone contains several ketone functional groups. Thus, Girard T reagent is usually used to derive cortisone to improve detection limit in MS analysis.[38,39] In this study, we selected these reactions as the model reactions to demonstrate the suitability of our approach in monitoring reaction species by MS in real time.

Experimental

Materials and reagents

Chemicals, including acethydrazide trimethylammonium chloride (Girard T reagent), phenyl hydrazine hydrochloride, formic acid, bradykinin, and cortisone were purchased from Sigma (St. Louis, MO, USA). Acetylacetone and acetonitrile were obtained from Fluka (St. Gallen, Switzerland). Sodium hydroxide and sodium chloride were obtained from J. T. Baker (Phillipsburg, USA). Capillaries (outer diameter: 361 μm; inner diameters: 50 μm) were obtained from Polymicro Technologies (Phoenix, AZ, USA).

Instrumentation

All the mass spectra were obtained in positive ion mode using an amaZon SL mass spectrometer (Bruker Daltonics, Bremen, Germany). The voltage set at the orifice of the mass spectrometer was −4500 V. The temperature of the ion transfer capillary was set to 200 °C-250 °C. The ion charge control function was on when conducting MS analysis, and the number of the collecting ions was set to 100,000. The maximum acquisition time was set to 200 ms. Each mass spectrum was obtained by averaging 10 mass spectra. The length of the metal extension tube adapted to the orifice of the mass spectrometer was ~4 cm, and the inner diameter of the tube was ~1 mm. A CD-4820 ultrasonicator (2.5 L, 160 W, 42 kHz) used in the UASI setup was purchased from Codyson (China).

On-line monitoring of chemical reactions

Scheme 1 shows the setup of the two-capillary-based UASI micro-reactor. Two capillaries were first conditioned by aqueous sodium hydroxide (1 N) for 20 min followed by rinsed with acetonitrile and deionized water (2:1, v/v) for 30 min using a water pump. The capillary was tapered by attaching a heavy object (50 g) on the low end of the capillary. A butane torch (RK2050 or RK2020 micro torch, Rekrow, Taiwan) was used to burn the polymer coating on the capillary surface near the heavy object for 2 cm. The tapered capillary

was cut by a triangle whetstone or ceramic. Two capillaries (length: 20 cm) used in the UASI setup were conditioned followed by filled with the required reagents and samples. The inlets of the two capillaries were placed in the vials containing the samples/reactants that were subjected into an ultrasonicator. The mass spectrometer and the ultrasonicator were switched-on initially, and the two UASI capillaries filled with reactants were placed ~2 mm away from the inlet of the mass spectrometer. The ions generated from each capillary were individually acquired by the mass spectrometer for 30 s to ensure the capillary function properly. Subsequently, the outlets of these two capillary were placed cross with an angle of ~60° close to the mass spectrometer to fuse the eluents. The gas phase ions resulting from the fusing of the eluents were simultaneously recorded by the mass spectrometer. When preparing 1,2-azole reactions, one capillary was filled with the phenylhydrazine (10^{-4} M) prepared in the solvent containing acetonitrile and deionized water (2:1, v/v) with 1% acetic acid, whereas the other capillary was filled with 0.1% acetylacetone prepared in the same solvent. When preparing on-line derivatization of cortisone using Girard T reagent as the derivatized reagent, one capillary was filled with Girard T reagent (5×10^{-4} M) prepared in the solvent containing acetonitrile and deionized water (2:1, v/v) with 1% acetic acid. The other capillary was filled with cortisone (5×10^{-5} M) prepared in the same solvent.

Preparation of urine samples

Cortisone was initially prepared in methanol and then diluted with the solvent of acetonitrile and deionized water (2:1, v/v) to a given concentration. Urine samples spiked with cortisone were used as simulated real samples. Urine samples collected from a healthy individual were centrifuged at 6000 rpm for 10 min (×2) followed by elimination of precipitates. The supernatant was collected and 50-fold diluted by the solvent containing acetonitrile and deionized water (2:1, v/v).

Results and Discussion

Factors affecting the flow rate of the analyte

The capillary tip diameter, capillary length, and the composition of the running solvent in UASI-MS analysis may affect the flow rate of analytes in the UASI capillary. Thus, we varied these abovementioned factors and monitored the flow rate of a model analyte, i.e. cortisone ($[M+H]^+$= 361.5), during UASI-MS analysis. The running solvent was filled with the UASI capillary first. After the UASI capillary inlet was put into the vial containing cortisone (50 μM), the mass spectrometer was switched on. **Figures S1A** and **S1B** show the extracted ion chromatographs (EICs) at *m/z* 361.5 obtained by varying the tip diameter to be ~11 μm and ~37 μm, respectively, with the same capillary length of 20 cm. The analyte ion at *m/z* 361.5 started to appear in the EICs **in Figure S1A** and **B** at the time points of 166 s and 31 s, respectively. The flow rates of the analyte in **Figure S1A** and **B** were estimated to be ~142 nL min^{-1} and ~761 nL min^{-1}, respectively. That is, the flow rate of the analyte was increased as the increase of the tip diameter. In addition, the length of the UASI capillary was varied. **Figures S1C** and **S1D** show the EICs at *m/z* 361.5 obtained by varying the capillary lengths to be 10 and 30 cm with a similar tip diameter (~11 μm). The analyte ion at *m/z* 361.5 started to appear in the EICs in **Figures S1C** and **D** at the time points at 68 s and 285 s, respectively. The flow rates of the analyte in **Figure S1C** and **S1D** were estimated to be ~260 nL min^{-1} and ~125 nL min^{-1}, respectively. The results indicated that the flow rate of the analyte was decreased as the increase of the capillary length. It is understandable because of the increase of the back pressure as the capillary length is extended. We further investigated the polarity of the running solvent in affecting analyte flow rate in UASI-MS analysis. **Figures S1E** and **S1F** show the EICs at *m/z* 361.5 obtained by varying the running solvent with the composition of acetonitrile and deionized water with the ratios of 2/1 and 1/1, respectively. The analyte ions started to appear in the EICs in **Figure S1E** and **S1F** at

the time points of 125 s and 173 s, respectively. The flow rates of the analyte in **Figure S1E** and **S1F** were estimated to be ~186 nL min^{-1} and ~135 nL min^{-1}, respectively. That is, the polarity of the running solvent can affect the flow rate in UASI-MS analysis. Furthermore, the running solvent with a lower polarity can allow the analyte to be eluted from the UASI capillary with a short time.

UASI-capillary-based micro-reactor for pyrazole synthesis reaction

As shown in **Scheme 1**, a cross section from the fusion of the eluents from these two capillaries placed ~1 mm away and angled at ~60° in front of the mass spectrometer was established. When the two capillaries were filled with reactants, the cross section acted as micro-reactor-like space and allowed the eluted reactants to interact upon fusion. **Figure 1** shows the photograph of the fused eluents derived from two UASI capillaries that were placed in front of the mass spectrometer. The fusing time was short because the solvent from the fused droplet quickly evaporated, and the resultant fine droplets derived from UASI processes were directed to the mass spectrometer immediately. As a result, the reaction was quickly terminated because the solvent evaporated and the resultant reacting species could be readily acquired by the mass spectrometer. This approach can be used to detect the ion species generated from fast reactions that can occur within sub-seconds. Furthermore, the reaction in the fused droplet can proceed faster than in a reaction vial because the concentration of the reactants can be raised to an extreme level upon solvent evaporation in the droplet.

We initially selected a pyrazole synthesis reaction as the model reaction. Phenylhydrazine and acetylacetone were selected as the reactants to conduct the reaction (**Figure 2A**). **Figure 2B** shows the extracted ion chromatographs (EICs) at *m/z* 109 (green curve) and *m/z* 173 (red curve) that were produced by the protonated phenylhydrazine and the reaction product (**Figure 2A**), respectively, obtained by introducing the mixture of the

reactants for conventional ESI–MS analysis. The flow rate was set to 200 μL/h (~3.3 μL/min), and the reaction species took ~0.5 min to reach the inlet of ESI ion source. The ion intensity at *m/z* 173 derived from the reaction product gradually increased over time. **Figure 2C** shows the corresponding mass spectrum obtained from 1–2 min. The peak at *m/z* 109 was derived from protonated phenylhydrazine, the peak at *m/z* 191 was derived from the intermediate, and the peak at *m/z* 173 was derived from the reaction product that appeared within the same mass spectrum. The intensities of the product and the intermediate ions were relatively low. The results indicated a slow progression of the reaction. UASI-based micro-reactors were then used to monitor the same reaction. **Figure 2D** shows the EICs at *m/z* 109 (green) and *m/z* 173 (red). During the first 30 s, only the capillary containing phenylhydrazine was placed in front of the mass spectrometer inlet. Subsequently, the capillary containing phenylhydrazine was removed, and the other capillary filled with acetylacetone was placed near the inlet of the same mass spectrometer during 30–60 s to ensure that the reagents eluted from these two capillaries could be observed separately. After 60 s, the capillary containing phenylhydrazine was returned to the front of the mass spectrometer inlet as shown in **Scheme 1A**. Apparently, the ion at *m/z* 109 derived from protonated phenylhydrazine (green) appeared during the first 30 s, whereas the ion derived from the reaction product at *m/z* 173 (green) appeared after 60 s. The product was generated immediately after the droplets, which were derived from the two capillaries containing phenylhydrazine and acetylacetone, fused. The ion intensity in **Figure 2D** is lower than that in **Figure 2B**. It was because that the flow rate at UASI (~ 200 nL/min) was 1 order of magnitude lower than that conducted in ESI–MS (~3.3 μL/min). **Figure 2E** shows the resultant mass spectrum obtained by acquiring the ions from 1.6–2.2 min. The peak at *m/z* 173 corresponding to the reaction product dominated the mass spectrum. Moreover, the peak at *m/z* 109 corresponding to the protonated phenylhydrazine and the peak at *m/z* 191 derived from the intermediate appeared in the same mass spectrum. The results

indicated that this UASI-based micro-reactor combined with UASI–MS analysis can react and detect chemical reactions simultaneously. The reaction species, including intermediates and products that proceeded within a few seconds, could readily be monitored by this UASI-MS approach. In addition, the results also indicated that the chemical reaction operated by the two capillary based UASI-MS approach can reveal product ions with a much shorter time than using conventional ESI-MS because of the concentration of the reactant dramatically increased during droplet fusion in UASI processes. Presumably, the slight vibration of the capillary resulting from the ultrasonication is the cause of the instability. Nevertheless, the instability does not affect the MS monitoring of the reaction species produced during the reaction.

Cortisone Derivatization by Girard T reagent

In addition, we selected cortisone, which can be used to suppress the immune system and reduce pain and inflammation,[44] as the model sample and used Girard T reagent as the derivatizing reagent to examine whether our UASI-based micro-reactor could be used to facilitate the reaction of cortisone and Girard T reagent for on-line UASI–MS analysis in real time. **Figure 3A** shows the reaction scheme occurring between Girard T reagent and cortisone. Initially, we used ESI–MS to examine whether the product ions could be observed in the mass spectra upon mixing these two reagents and continuously injecting to the mass spectrometer. **Figure 3B** shows the EICs at *m/z* 132 (green) derived from Girard T reagent, the ion at *m/z* 361 (blue) derived from protonated cortisone, and the product ion at *m/z* 474 (red). Apparently, the ion intensity derived from the two reactants remained constant, whereas the intensity of the red curve remained zero, indicating that no ions were derived from the reaction product. **Figure 3C** shows the corresponding mass spectrum acquired from 14–15 min. The peak at *m/z* 132 dominated the mass spectrum, and a weak peak at *m/z* 361 was observed in the same mass spectrum. However, the product ion peak at *m/z* 474 was not

observed. The reaction time might have been insufficient to reveal the product ion. Thus, the reaction of derivatizing cortisone by Girard T reagent was reacted under 1-h vortex mixing prior to ESI–MS analysis. However, the MS results were similar to those obtained in **Figures 3B** and **3C**. **Figure 3D** shows the resultant mass spectrum that was dominated by the ion at *m/z* 132 and *m/z* 361. The results indicated that cortisone reacted slowly with Girard T reagent. When the reaction was conducted for 1 h, the product ion was not observed in the resultant ESI mass spectra. We further used our setup to conduct the reaction by reacting cortisone and the Girard T reagent in the UASI-based micro-reactor. **Figure 3E** shows the EICs at *m/z* 132 (green), *m/z* 361 (blue), and *m/z* 474 (red). From 0–0.6 min, we only placed the UASI capillary containing Girard T reagent near the mass spectrometer inlet. Thus, only the ion intensity derived from Girard T reagent (green curve) was observed (**Figure 3E)**. After 0.6 min, a capillary containing cortisone was placed in front of the mass spectrometer inlet, and its eluent was fused with that from the other capillary containing Girard T reagent. The ion intensity derived from cortisone (blue) started to appear in the EIC (**Figure 3E**). After 1.3 min, the reagents eluted from the two capillaries were placed properly to allow for eluent fusion. **Figures 3F** and **3G** show the resultant mass spectra acquired from 2.0–2.5 and 3.0–3.5 min, respectively. The product ion at *m/z* 474 was observed in the mass spectrum in addition to the peaks derived from the reactants at *m/z* 132 and *m/z* 361. The product ion peak at *m/z* 474 appeared after fusing the eluents from the two capillaries for 1 min. Presumably, the reactant concentrations in the UASI-based microreactor were dramatically increased because of solvent evaporation. The reaction product could therefore be observed within 1 min. Moreover, the ion intensity increased as the monitoring time increased, indicating that the reactant can be retained in the micro-reactor to a certain extent. In addition, the ion intensity was relatively low and unstable compared with those observed using ESIMS. It was because that the reaction could only be undergone upon the fusion of the reactant droplets

eluted from the UASI capillaries. Furthermore, the flow rate of the reactants eluted from the UASI capillaries was very low. Thus, the ion intensity is instable and low. Nevertheless, the results indicated that the UASI-based micro-reactor could allow the reaction product derived from the reaction of cortisone and the Girard T reagent to be observed within ~1 min. Furthermore, the UASI setup can be readily facilitate the ionization of the reaction species.

In addition, we also varied the cross angle between the two UASI capillaries to examine whether the angle can affect the reaction rate. **Figure S2** shows the summarized results obtained by plotting the reaction time required to obtain product ion at *m/z* 474 in the current UASI approach using the same reaction as shown in **Figure 3** versus the angle. The reaction time was in the range of 40–60 s when varying the angle from 30 to 150°. However, as the angle was increased to 120°, the reproducibility of the reaction time became worse. Such an effect can be clearly observed from the error bars shown in **Figure S2**. The error bar became bigger as the angle was increased. Thus, the suitable cross angle is in the range of 30-60°. Moreover, we also investigated whether the distance between the fused droplet created microreactor and the inlet of the mass spectrometer would affect the reaction rate. Our results showed that the reaction time for obtaining product ions in the UASI mass spectrum was shorter when placing the fused microdroplet to the MS inlet with the distance of ~1 mm than that of ~5 mm. It took ~60 s and ~80 s to observe the product ions appearing in the UASI mass spectra when the distances between the microreactor and the inlet of the mass spectrometer were ~1 mm and ~5 mm, respectively. We found that electrospray frequency was higher when the distance was ~1 mm (**Supplementary video 1**) than ~5 mm (**Supplementary video 2**). Presumably, the fused microdroplet could accumulate charges faster to form the Taylor cone at the distance of ~1 mm than ~5 mm between the microreactor and the inlet of the mass spectrometer. The difference of the electrospray frequency might also be the reason why the reaction rate at the distance of ~ 1 mm was higher than that of ~ 5

mm. In addition, the ion signal is more stable with the distance of ~1 mm than with that of ~5 mm.

In addition, the entire experiments had been repeated by two co-authors. When the cross angle of the two UASI capillaries was adjusted to 60°, the times required to observe the appearance of the product obtained from two individual experiments conducted by two co-authors were ~59 s and ~60 s, respectively. Thus, the reproducibility was acceptable.

One may wonder if the results may be improved if the UASI capillary outlets are applied with any electric contact such as grounded. Thus, we applied grounding on the inlets of the two UASI capillaries and conducted the same reaction as shown in **Figure 3**. The reaction can still be carried out and the product ion can also be observed in the resultant mass spectra. However, the ion intensity resulting from the reaction species slightly decreased with increased background ions. It is understandable because applying direct electric-contact on the capillary outlet can lead to generation of solvent background ions through electrochemical reaction. It is also the reason why we usually did not apply any electric contact directly on the emitter in our approaches.[22-25, 39, 41-43] That is, to apply any electric contact on the capillary outlets is not necessary. Using a simpler setup can obtain better results. Moreover, the factor contributed by applying electric contact on the reactant eluents that can affect the reaction is eliminated in our current approach.

Cortisone derivatization by Girard T reagent in urine samples

The results above have demonstrated the feasibility of using our setup to monitor the reaction of cortisone with Girard T reagent using the proposed UASI-based setup. The reaction proceeded quickly, and the resultant species was detected simultaneously by the mass spectrometer in real time. Considering that cortisone is usually present in samples such as urine containing complex matrices, the ion derived from cortisone can be easily suppressed during MS analysis if the sample pretreatment is not done properly prior to MS analysis. As

mentioned earlier, the pre-charged property of Girard T reagent can improve the ionization efficiency of the cortisone derivatives in MS analysis. Furthermore, the reaction occurring between Girard T reagent and cortisone allows cortisone can be selectively detected from complex samples owing to the improved the ionization efficiency. Thus, we used a urine sample spiked with cortisone as the model sample to examine the possibility of using our setup to selectively detect cortisone from complex urine samples. As mentioned earlier, cortisone has been used as the medicine to reduce pain and inflammation. For a healthy adult, the allowed daily dose of cortisone is 25–300 mg.[44] Considering the volume of urine that an adult urinates daily is 750-2000 mL,[45] the concentration of cortisone derived metabolites in urine was estimated to be in the range of 4.60×10^{-5} M–5.55×10^{-4} M. On the basis of the estimation, 50-fold diluted urine samples spiked with cortisone with the concentrations of 9.2×10^{-7} M and 1.1×10^{-5} M were used as the model samples**. Figure 4A** shows the direct mass spectrum of the urine sample without sample pretreatment. The peaks at *m/z* 361 and 383 were presumably derived from protonated and sodiated adducts of cortisone, respectively. The peak at *m/z* 114 was derived from creatinine, which is a common metabolite present in urine, can be used as the internal standard. We further used our UASI-based micro-reactor to confirm the identity by adding Girard T reagent (5×10^{-4} M) into one UASI capillary. **Figure 4B** shows the resultant mass spectrum. One new peak exists at *m/z* 474, derived from the derivatized cortisone by the Girard T reagent in the mass spectrum. The results indicated that we can use this simple micro-reactor on-line MS detection approach to selectively derivatize target analytes from complex samples. When the concentration of the cortisone spiked in urine was lowered to 5×10^{-7} M, the ions derived from cortisone and its derivatized product were not observed in the resultant mass spectrum (**Figure 4C**). To further improve the result, we used MS/MS mode by selecting the peak at *m/z* 474, representing the product ion as the precursor ion, to monitor the same reaction (**Figure 4D**). The ion peaks at *m/z* 444, 415, and

387 corresponding to the losses of -CH_2O, -$N(CH_3)_3$, and -$(CH_3)_3NCO$, respectively (**Figure 4D**). These results were very similar to that reported in the previous study,[40] which concluded that chemical reactions can be accelerated in charged droplets. Although there was no direct electric contact made on the UASI capillary, the eluent droplets were polarized and carried charges owing to polarization effect[41,42] that was induced by the high electric field provide by the mass spectrometer. These results demonstrate the feasibility of using our current method to selectively react cortisone from complex urine samples. It was possible to confirm the presence of cortisone from complex samples by detection of its derivatized product ions. However, to further improve the detectable concentration should be done. We believe if sample pretreatment by removing most of impurities such as salt interferences from complex samples can be conducted prior to MS analysis, the detectable concentration can be further decreased.

Conclusions

In this study, we have successfully demonstrated that a micro-reactor based on the use of the UASI setup can be used to conduct fast reactions and monitor the reaction species on-line by UASI–MS. Sprays derived from the eluents of the two UAS capillaries filled with different reactants were fused to carry our chemical reactions *in situ* near the inlet of the mass spectrometer. Moreover, our results demonstrated that the reaction in the UASI-based micro-reactor could be sped up owing to the fast solvent evaporation in the fused droplets in the micro-reactor. Therefore, the reactant concentrations were thoroughly increased, and the reaction species were easily observed in the resultant mass spectra. The reagent consumption in this approach was minimized because only a few microliters of reagents are sufficient to perform the reaction in the developed micro-reactor. This setup only requires low amount of samples and can be easily set up in front of the mass spectrometer. Our current approach may be feasible for investigating intermediates with short lifetimes and facilitating the understanding of mechanisms behind certain chemical reactions. Compared with other existing approaches that use fused droplets for conducting reactions in situ developed by other research groups, our approach does not require any direct electric contact to the fused droplets containing reactants. Therefore, the factor of the electric voltage that may affect reactions monitored in real time can be eliminated. Thus, our setup should benefit many areas, including reaction monitoring and *in situ* increasing ionization efficiency of target analytes.

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References

(1) Z. T. Cygan, J. T. Cabral, K. L. Beers, E. J. Amis, Microfluidic platform for the generation of organic-phase microreactors. *Langmuir.* **2005**, *21*, 3629-3634.

(2) H. Song, D. L.Chen, R. F. Ismagilov, Reactions in droplets in microfluidic channels. *Angew Chem Int Ed Engl.* **2006**, *45*, 7336-7356.

(3) J. Gao, J. Xu, , L. E. Locascio, C. S. Lee, Integrated microfluidic system enabling protein digestion, peptide separation, and protein identification. *Anal. Chem.* **2001**, *73*, 2648-2655.

(4) L. J. Jin, J. Ferrance, J. C. Sanders, J. P. Landers, A microchip-based proteolytic digestion system driven by electroosmotic pumping. *Lab Chip.* **2003**, *3*, 11-18.

(5) D. C. Duffy, J. C. McDonald, O. J. Schueller, G. M. Whitesides, Rapid prototyping of microfluidic systems in poly (dimethylsiloxane). *Anal. Chem.* **1998**, *70*, 4974-4984.

(6) H. Sharma, D. Nguyen, A. Chen, V. Lew, M. Khine, Unconventional low-cost fabrication and patterning techniques for point of care diagnostics. *Ann Biomed Eng.* **2011**, *39*, 1313-1327.

(7) M. Yamashita, J. B. Fenn, Electrospray ion source. Another variation on the free-jet theme. *J. Phys. Chem.* **1984**, *88*, 4451-4459.

(8) M. Yamashita, J. B. Fenn, Negative ion production with the electrospray ion source. *J. Phys. Chem.* **1984**, *88*, 4671-4675.

(9) J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, C. M. Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules. *Science* **1989**, *246*, 64-71.

(10) R. Ramsey, J. M. Ramsey, Generating electrospray from microchip devices using electroosmotic pumping. *Anal. Chem.* **1997**, *69*, 1174-1178.

(11) Q. Xue, F. Foret, Y. M. Dunayevskiy, P. M. Zavracky, N. E. McGruer, B. L. Karger, Multichannel microchip electrospray mass spectrometry. *Anal. Chem.* **1997**, *69*, 426-430.

(12) J. S. Kim, D. R. Knapp, Miniaturized multichannel electrospray ionization emitters on

poly (dimethylsiloxane) microfluidic devices. *Electrophoresis.* **2001**, *22*, 3993-3999.

(13) I. M. Lazar, R. S. Ramsey, S. Sundberg, J. M. Ramsey, Subattomole-sensitivity microchip nanoelectrospray source with time-of-flight mass spectrometry detection. *Anal. Chem.***1999**, *71*, 3627-3631.

(14) W. C. Sung, H. Makamba, S.-H. Chen, Chip-based microfluidic devices coupled with electrospray ionization-mass spectrometry. *Electrophoresis.* **2005**, *26*, 1783-1791.

(15) C.-H. Chang, T.-Y. Chen, Y.-C. Chen, Microfluidic chip coupled with thermal desorption atmospheric pressure ionization mass spectrometry. *Mass Spectrom (Tokyo).* **2014**, *3*, S0026.

(16) J. Li, P. Thibault, N. H. Bings, C. D. Skinner, C. Wang, C. Colyer, J. Harrison, Integration of microfabricated devices to capillary electrophoresis-electrospray mass spectrometry using a low dead volume connection: application to rapid analyses of proteolytic digests. *Anal. Chem.***1999**, *71*, 3036-3045.

(17) B. Zhang, H. Liu, B. Karger, F. Foret, Microfabricated devices for capillary electrophoresis-electrospray mass spectrometry. *Anal. Chem.* **1999**, *71*, 3258-3264.

(18) D.-Y. Chang, C.-C. Lee, J. Shiea Detecting large biomolecules from high-salt solutions by fused-droplet electrospray ionization mass spectrometry. *Anal Chem.* **2002**, *4,* 2465-2469.

(19) H.-W. Chen, A. Venter, R.G. Cooks, Extractive electrospray ionization for direct analysis of undiluted urine, milk and other complex mixtures without sample preparation. *Chem. Commun*. **2006**, *19*, 2042-2044.

(20) L. Zhu, G. Gamez, H.-W. Chen, H.-X. Huang, K. Chingin, R. Zenobi
Real-time, on-line monitoring of organic chemical reactions using extractive electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom. **2008**, *19*, 2993-2998.

(21) J.K. Lee, S. Kim, H. G. Nam, R. N. Zare. Microdroplet fusion mass spectrometry for fast reaction kinetics. *Proc Natl Acad Sci U S A.* **2015**, *112,* 3898-3903.

(22) T.-Y. Chen, J.-Y. Lin, J.-Y. Chen, Y.-C. Chen, Ultrasonication-assisted spray ionization

mass spectrometry for the analysis of biomolecules in solution. *J. Am. Soc. Mass. Spectrom.* **2010**, *21*, 1547-1553.

(23) S.-H. Lin, T.-J. Lo, F.-Y. Kuo, Y.-C. Chen, Real time monitoring of accelerated chemical reactions by ultrasonication-assisted spray ionization mass spectrometry. *J. Mass Spectrom*. **2014**, *49*, 50-56.

(24) T.-J. Lo, T.-Y. Chen, Y.-C. Chen, Study of salt effects in ultrasonication-assisted spray ionization mass spectrometry. *J. Mass Spectrom*. **2012**, *47*, 480-483.

(25) T.-Y. Chen, C.-S. Chao, K.-K. T. Mong, Y.-C. Chen Ultrasonication-assisted spray ionization mass spectrometry for on-line monitoring of organic reactions. *Chem. Commun*. **2010**, *46*, 8347-8349.

(26) C. Wiles, P. Watts, S. J. Haswell, E. Pombo-Villar, The application of microreactor technology for the synthesis of 1, 2-azoles. *Org. Process Res. Dev.* **2004**, *8*, 28-32.

(27) E. Cordes, W. Jencks, On the mechanism of Schiff base formation and hydrolysis. *J. Am. Chem. Soc.* **1962**, *84*, 832-837.

(28) J. S. Larsen, M. A. Zahran, E. B. Pedersen, C. Nielsen, Synthesis of triazenopyrazole derivativesas potential inhibitors of HIV-1. *Monatsh. Chem.* **1999**, *130*, 1167-1173.

(29) H. Katayama, T. Oshiyama, Preparation and bioactivity of pyrazole derivatives as potential cross-linking agent. *Can. J. Chem*. **1997**, *75*, 913-919.

(30) D. M. Bailey, P. E. Hansen, A. G. Hlavac, E. R. Baizman, J. Pearl, A. F. DeFelice, M. E. Feigenson, 3, 4-Diphenyl-1H-pyrazole-1-propanamine antidepressants. *J Med Chem.* **1985**, *28*, 256-260.

(31) Z.-X. Wang, H.-L. Qin, Solventless syntheses of pyrazole derivatives. *Green Chem.* **2004**, *6*, 90-92.

(32) D. W. Johnson, Ketosteroid profiling using Girard T derivatives and electrospray ionization tandem mass spectrometry: direct plasma analysis of androstenedione, 17-

hydroxyprogesterone and cortisol. *Rapid Commun Mass Spectrom.* **2005**, *19*, 193-200.

(33) O. H.Wheeler, The Girard reagents. *J. Chem. Edu.* **1968**, *45*, 435-437.

(34) O. H. Wheeler, The Girard reagents. *Chem. Rev*. **1962**, *62*, 205-221.

(35) H. P. Ehrlich, T. K. Hunt, Effects of cortisone and vitamin A on wound healing. *Ann. Surg.* **1968**, *167*, 324.

(36) J.-S. Raul, V. Cirimele, B. Ludes, P. Kintz, Detection of physiological concentrations of cortisol and cortisone in human hair. *Clin. Biochem.* **2004**, *37*, 1105-1111.

(37) J. Polson, C. Pattee, F. Woolhouse, Keloid. *Can. Med. Assoc. J.* **1951**, *65*, 447-449.

(38) M. Girod, E. Moyano, D. I. Campbell, R. G. Cooks, Accelerated bimolecular reactions in microdroplets studied by desorption electrospray ionization mass spectrometry. *Chem. Sci.* **2011**, *2*, 501-510.

(39) S.-H. Lin, T.-J. Lo, F.-Y. Kuo, Y.-C. Chen, Real time monitoring of accelerated chemical reactions by ultrasonication-assisted spray ionization mass spectrometry. *J. Mass Spectrom*. **2014**, *49*, 50-56.

(40) A. K. Badu-Tawiah, D. I. Campbell, R. G. Cooks, Reactions of microsolvated organic compounds at ambient surfaces: droplet velocity, charge state, and solvent effects. *J. Am. Soc. Mass. Spectrom.* **2012**, *23*, 1077-1084.

(41) A. K. Meher, Y-C. Chen, Polarization induced electrospray ionization mass spectrometry for the analysis of liquid, viscous, and solid samples. *J. Mass Spectrom.* 2015, 50, 444-450.

(42) A. K. Meher, Y.-C. Chen. Online monitoring of chemical reactions by polarization-induced electrospray ionization. *Anal. Chim. Acta.* 2016, 937, 106-112.

(43) M.-L. Wu, T.-Y. Chen, Y.-C. Chen, Y.-C. Chen Carbon fiber iIonization mass spectrometry for the analysis of analytes in vapor, liquid, and solid phases. *Anal. Chem.* 2017, 89, 13458-1346.

(44) https://www.drugs.com/dosage/cortisone.html (retrived on 2018-10-06).

(45) Estridge, B. H.; Reynolds, A. P.; Walters, N. J. In *Basic Medical Laboratory Techniques*, 4th ed.; Delmar Publishers: Albany, NY, 2000; pp 333.

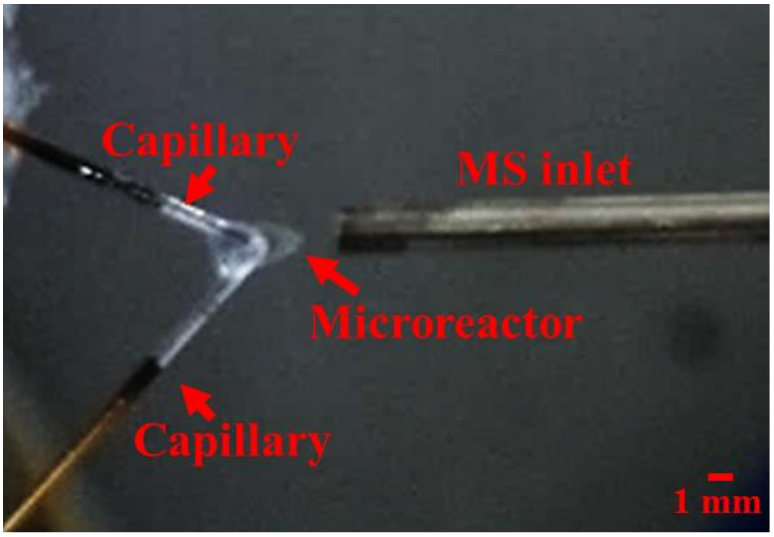


Figure 1. Photograph of the fused droplet from the two capillary-based UASI microreactor that is set up in front of the inlet of the mass spectrometer.

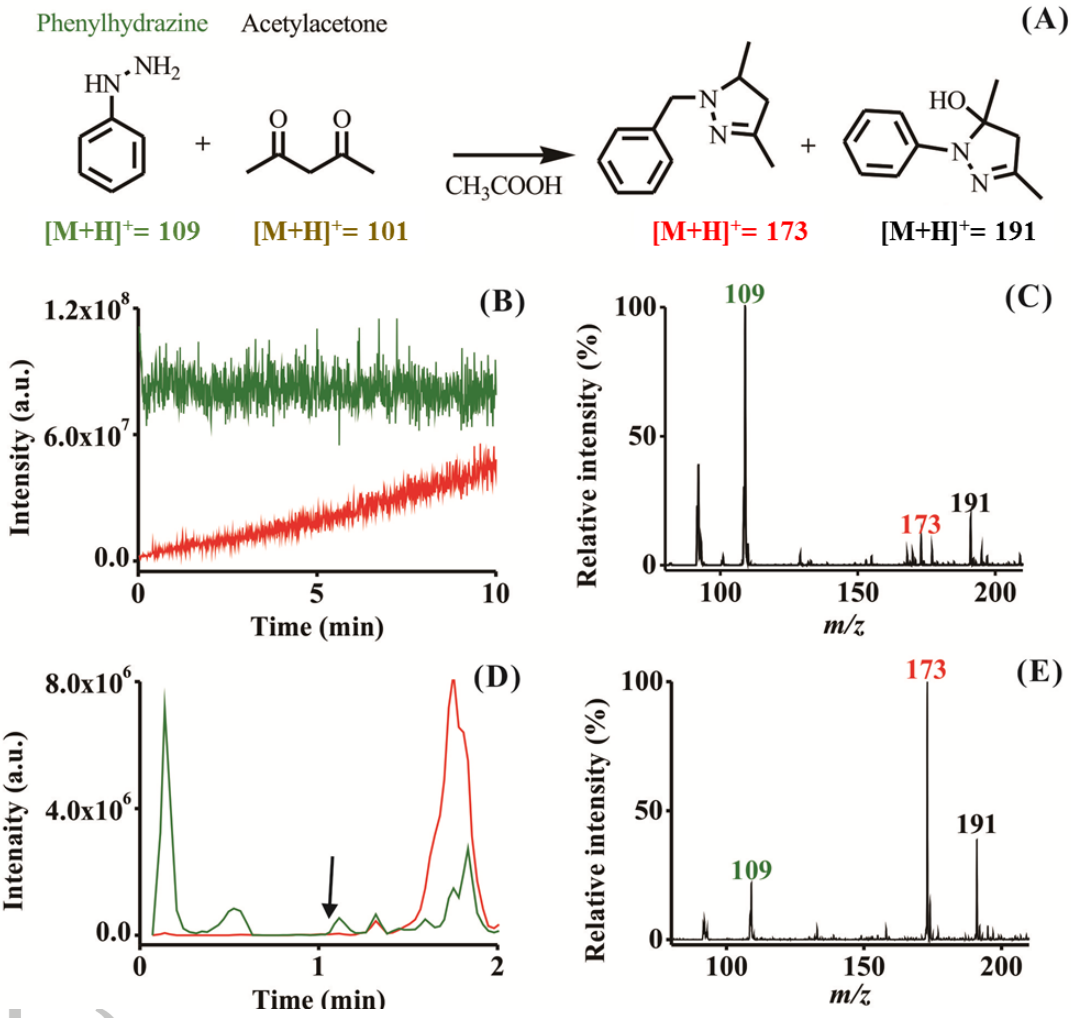


Figure 2. (A) Reaction scheme of phenylhydrazine and acetylacetone. The reaction was conducted by fusing the eluents from two capillaries, in which one contained phenylhydrazine (10^{-4} M) prepared in the solvent of acetonitrile and deionized water (2:1, v/v) containing acetic acid (1%) and the other one contained acetylacetone (0.1%) prepared in the same solvent. (B) EICs at *m/z* 109 (green) and *m/z* 173 (red) obtained by using ESI–MS as the detection method and (C) its corresponding mass spectrum acquired from 1 to 2 min. The inner diameter of the ESI emitter was ~400 μm. (D) EICs at *m/z* 109 (green) and *m/z* 173 (red) obtained by using the two-capillary-based UASI micro-reactor for conducting reaction on-line and (E) its corresponding mass spectrum acquired from 1.6 to 2.2 min. The arrow in

Panel D indicated the time point that the two capillaries were put cross at the angle of ~60^{o} in front of the inlet of the mass spectrometer. The inner diameter of the capillaries was ~50 μm, while the tip diameter of the capillaries was ~10 μm.

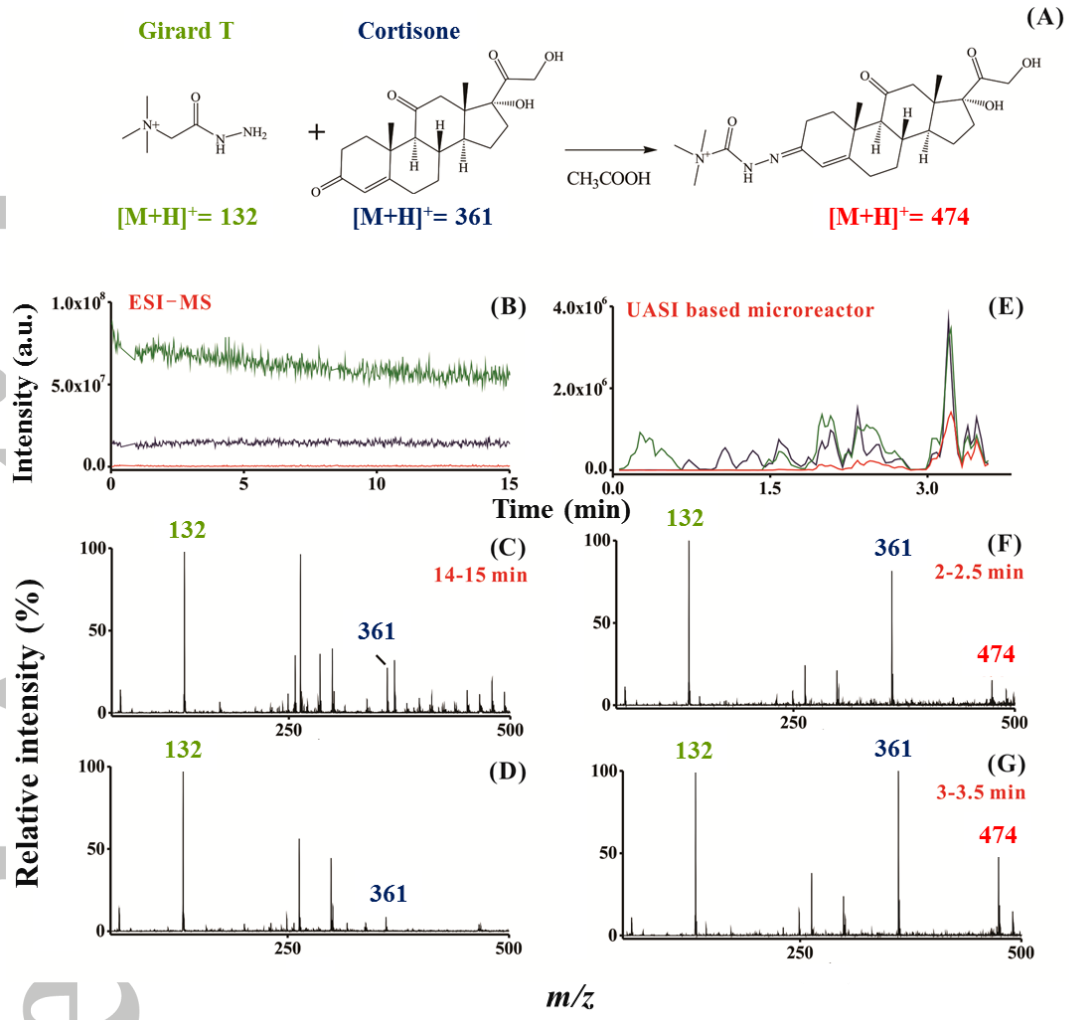


Figure 3. (A) Reaction scheme of Girard T reagent with cortisone. The concentration of Girard reagent T (*m/z* 132) was 5 ×10^{-4} M, and the concentration of cortisone (*m/z* 361) was 5 × 10^{-5} M. (B) EICs at *m/z* 132, *m/z* 361, and *m/z* 474 obtained from the sample containing the two reactants and using ESI–MS as the detection tool. (C) The resultant mass spectrum acquired from 14 to 15 min. The reactants were mixed right before conducting ESI-MS analysis. The flow rate of the sample injection was set at 200 µL/h. (D) ESI-mass spectrum of the sample that was obtained after vortex-mixing the two reactants for 1 h. (E) EICs at *m/z* 132, *m/z* 361, and *m/z* 474 obtained from the reaction taking place in the UASI based micro-reactor, in which two capillaries were filled with two different reactants followed by fusing the eluents with an angle of ~60º in front of the inlet of mass spectrometer. (F) The resultant mass spectra acquired from 2.0 to 2.5 min and (G) 3.0 to 3.5 min.

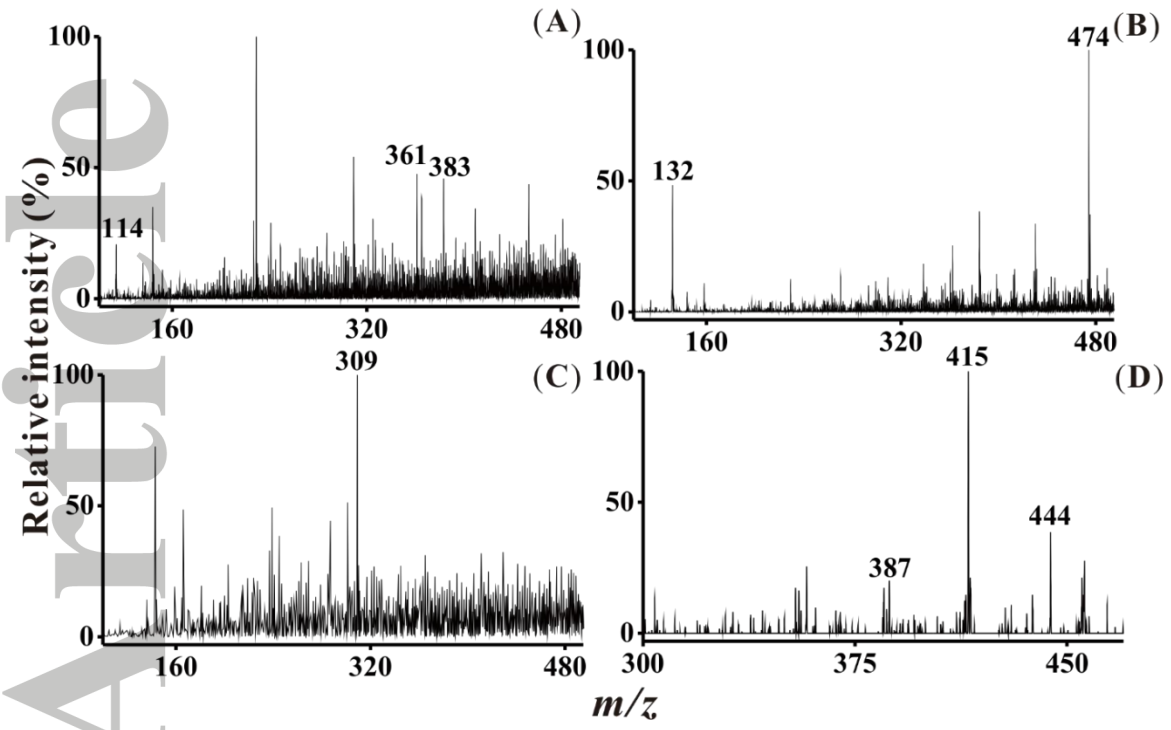
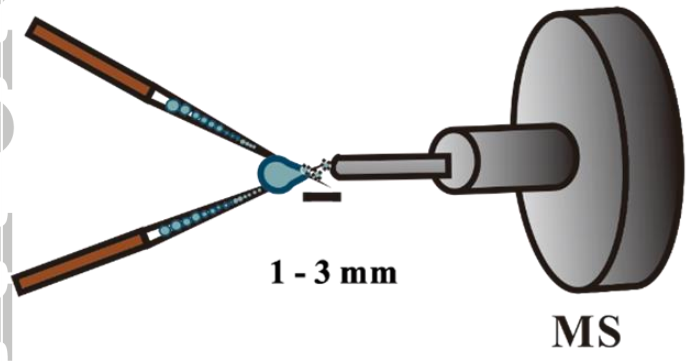


Figure 4. (A) UASI mass spectrum of the urine sample spiked with cortisone (10^{-5} M). (B) Mass spectrum of the sample containing cortisone (10^{-5} M) that was derivatized *in situ* in the UASI capillary-based microreactor and monitored by UASI-MS on-line. (C) UASI mass spectrum of the urine sample spiked with cortisone (5×10^{-7} M). (D) MS/MS spectrum obtained by selecting the ion at *m/z* 474 as the precursor ion derived from the sample containing cortisone (5×10^{-6} M) that was derivatized *in situ* in the UASI capillary-based microreactor and monitored by UASI-MS on-line.

Scheme 1. Cartoon illustration of the setup of the two UASI-capillary–based micro-reactor for *in situ* chemical reaction and simultaneous UASI-MS analysis. Two USAI capillaries were placed cross with an angel of ~60^{o} in proximity to the inlet of the mass spectrometer.



Graphical Abstract