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Untreated and Dried Sample Analysis by Solid Probe Assisted Nano Electrospray Ionization Mass Spectrometry

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Abstract:

The present paper describes the application of solid probe assisted nano electrospray ionization mass spectrometry (SPA-nanoESI-MS) for the direct analysis of samples in solid or dried form. The experimental procedure is simple and requires a metallic needle to touch the sample surface followed by inserting the needle to a solvent preloaded nano-capillary. A number of real-world samples in solid or dried form comprising proteins, drugs in tablets, illicit drug in dried urine, lipids in tissues were analyzed to evaluate the applicability of the technique in diverse fields. The technique can produced high quality mass spectra without clogging the capillary tip. Semiquantitative aspect of this technique was evaluated using morphine from dried urine. In addition, biofluids/biomolecules captured on the needle tip and stored for several days at room temperature produced almost similar spectra compared to the fresh samples for cancerous and noncancerous tissues. The high sensitivity, versatile applicability and capability of dried sample analysis extend the scope of SPA-nanoESI to new ventures like bioanalytical and forensic analysis as well as clinical diagnosis.

Introduction:

Mass spectrometry (MS) is one of the most sensitive analytical techniques having wide applicability in complex sample analysis with high specificity.[1,2] This technique is highly adoptable for both qualitative and quantitative studies in diverse fields.[1–3] Gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) are the preferred MS based techniques for forensic and regulatory laboratories where a large number of samples need to be analyzed in rapid and reliable basis.[4–7] Both GC-MS and LC-MS require time consuming chromatographic separations and the real-world samples such as urine and biological tissues need to undergo liquid-liquid or solid phase extraction prior to the analysis.[4–7]

Ambient mass spectrometry based techniques and their applications are favorably increasing since the development of desorption electrospray ionization (DESI) and Direct analysis in real time (DART). These techniques overcome most of the precautions (extraction, pre-concentration, derivatization, etc.) crucial for conventional GC-MS or LC-MS analysis and get exceptional access to analyze the raw biological samples directly or with minimal pre-treatment.[8–10] DESI is basically a spray based direct desorption and ionization technique [11] and DART is a plasma based ionization technique,[12] which have been used for rapid onsite sample screening from complex matrices with sufficient specificity and sensitivity. Chiefly the high tolerance to impurities, matrices and high speed scanning with adequate specificity and sensitivity led the ambient MS techniques particularly useful for rapid and reliable screening of illicit drugs from body fluids[13–15] and components of biological tissues in dried, frozen and fresh forms.[16] In addition, conventional electrospray ionization (ESI) technique has been used as an auxiliary ionization source, e.g., electrospray-assisted laser desorption/ionization (ELDI),[17] laser ablation with electrospray ionization (LAESI),[18] matrix assisted laser desorption electrospray

ionization (MALDESI)[19] and laser-induced acoustic desorption electrospray ionization (LIAD/ESI)[20] to analyze untreated and dried sample directly from the surface.

The use of biological tissues as a direct electrospray emitter has also been reported in tissue spray,[21,22] leaf spray,[23] and needle biopsy/direct electrospray from the biopsied tissue.[24] But solvents were applied on the sample surface in all of the above mentioned methods to extract the target analytes and produce a stable ESI beam. A simple spot sampling method was developed by Van Berkel et al. using a liquid microjunction surface sampling probe/electrospray ionization mass spectrometry which has been used for direct biological tissue analysis from the dried and frozen surface.[25,26] Recently, Roach et al. reported a method called nanospray desorption electrospray ionization (Nano-DESI) for liquid-extraction surface sampling in mass spectrometry using similar approach.[27] Additionally, several cost effective techniques such as paper spray,[28] touch spray,[29] wooden-tip ESI,[30] Al-foil ESI,[31] and pipette-tip ESI[32–35] were reported in recent time for the real world sample analysis.

In 2007 probe electrospray ionization mass spectrometry (PESI-MS) was developed using a solid needle.[36] Prior to this development, ESI was also performed from a solid metal probe by Hong et al.[37] The novel feature of electrospray generated from solid needle/probe is that it produces the discontinuous sampling namely sequential and exhaustive ionization.[38] Therefore, PESI-MS can be applied to protein and peptides analysis with high-concentration salts and detergents solution.[39,40] Due to ease in handling, PESI has been applied to cancer diagnosis,[41–44] imaging,[45] plant compound analysis[46], organometallic compound analysis,[47] and narcotics monitoring from complex biomaterials.[48] PESI has also been applied to the solvent free green chemistry,[49] and single cell analysis.[50] For the real time and direct chemical constituents analysis from dry and real-world samples, a sheath-flow probe electrospray ionization mass spectrometry

(SF-PESI-MS) has been developed.[51,52] A novel ambient ionization method namely Leidenfrost phenomenon assisted thermal desorption (LPTD) coupled to dielectric barrier discharge (DBD) ionization mass spectrometry has been developed recently and applied for identifying the trace level of narcotics and anabolic steroids in urine.[53,54]

For the direct micro-extraction and sampling for mass spectrometry, solid probe assisted nano-electrospray ionization (SPA-nanoESI) was also developed.[55] In SPA-nanoESI, sample was extracted using a metallic needle tip by sticking it to the biological tissue and then the needle was inserted into the solvent-preloaded nanoESI capillary from solvent loading side and electrospray was generated by applying voltage on nanoESI capillary.[55] Alternatively, a gel loading pipette tip was used instead of nanoESI capillary to facilitate the direct and selective analysis and cancer diagnosis.[33] In this study SPA-nanoESI was applied for the direct analysis of dried/powder narcotics, drug tablets and narcotic screening from dried raw urine. Further, the technique was extended to analyze the biomolecules from dried biological tissues.

Solvents and Reagents

All solvents and reagents used in this study were of analytical grade or higher and were used without further purification. Water was purified by a Milli-Q system (Millipore, Bedford, MA). High-performance liquid chromatography (HPLC) grade organic solvents were purchased from Kanto Chemicals (Tokyo, Japan). All protein standards were obtained from Sigma-Aldrich (Tokyo, Japan).

Sample Preparation

The urine samples were prepared by spiking 1 mL of raw urine with 10 μL of the stock solution of morphine (50 mg/ml).The spiked sample was further diluted directly in raw urine without using any solvent. Kidney tissues were about 10 × 4 × 2 mm^3 in size. Cancer tissues from patients diagnosed as clear cell renal cell carcinoma (ccRCC) and their uninvolved kidney tissues were obtained from the Department of Surgery, Faculty of Medicine, University of Yamanashi. All the procedures relating to the handling of human specimens were approved by the Ethical Committee of the Faculty of Medicine, University of Yamanashi. Appropriate precautions should be taken when handling cancerous biomaterials.

Experimental

The experimental procedure was similar to our previous study (SPA-nanoESI) and is shown schematically in Fig 1. In brief, a 0.3 mm acupuncture needle with submicron tip diameter was touched to the powder sample by dipping in 1-2 mm or stuck into the tablet. The sample amount can be determined by depositing absolute amount of the analytes in solution on the needle tip. 500 nl sample was loaded by gel loading pipette tip on the needle tip and waited for complete drying. To deposit the sample solution easily a sample piece of chromatography paper (~2 x 0.5 x 0.18 mm^3) could be stuck to the needle tip. Additionally, after biological tissue sampling, the needle was stored in a glass vial at room temperature for several days for representing as dried real world biomaterials and served for the measurements. After sampling, the needle was inserted into a nanoESI capillary, in which 2 μL of organic solvent (e.g., acetonitrile, methanol or iosopropanol) was preloaded. The electrospray duration for 2 μL solution was about 20 min,[55] but the analysis time could be a few sec. The nanoESI capillary was then positioned at a distance

of 5 mm in front of the inlet of a mass spectrometer. High voltage (0.5-1.5 kV) was applied to the metal coated part of the nanoESI capillary for electrospray. Alternatively high voltage can be applied to the needle that produces exactly same mass spectra. The experiments were performed at least three times to ensure the excellent reproducibility.

Results and Discussion

Surface analysis techniques such as infrared (IR)[56], near-infrared (NIR)[57,58], Raman[59] and fluorescence[60,61] spectroscopy have normally been applied for the fast screening of unknown tablets in solid forms as sample preparation and separation are not required.[62] But the sensitivity and reliability of these techniques are much lower compared to the mass spectrometry. In 2006, Leuthold et al. reported the usefulness of drug analysis in emergency departments of hospitals or police department where patients and addicted persons arrive with unknown tablets and powders in their pockets and a rapid analysis of the tablets could save precious time for diagnostics.[62] Powder narcotics can readily be analyzed using SPA-nanoESI by simply touching the powder or tablets using a needle tip and then inserting the needle to the solvent preloaded nanoESI capillary. Fig. 2 illustrates mass spectrum obtained from opium powder. Opium is the dried latex from the opium poppy (*Papaver somniferum*) seed pod. Several alkaloids including codeine, morphine, thebaine, parpavrine, noscapine, and corydaline are detected from opium powder shown in Fig.2. The alkaloids[63] were identified by their exact masses using Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Supplementary Fig. 1(a-d) shows the mass spectra of other narcotics such as methamphetamine, cocaine, morphine, and codeine, respectively.

A standard morphine solution as well as morphine-spiked urine was used to calculate the precision, linear ranges, correlation coefficient (R^2), and recovery of this technique. In all cases, calculations were made based on the intensity, i.e., the intensity versus the deposited sample amount on the needle tip. In order to mount the known amount of sample at the needle tip, 500 nL of aqueous sample solution was deposited at the needle tip using a gel loading pipette tip and dried at room temperature. Sample could also be loaded at the tip of the needle using a chromatography paper. In this case, a small piece of chromatography paper was first attached to the needle tip and soaked with the sample solution and dried and then the needle with chromatography was inserted to the solvent preloaded nanoESI capillary. This procedure facilitates the transition of the samples trapped in the chromatography paper to the nano capillary.

10 to 5000 pg of morphine (20 ppb to 10 ppm in solution) were deposited on the needle tip to examine the linear dynamic range of the SPA-nanoESI. Under the optimized experimental conditions, the LOD was found to be 10 pg. Supplementary Fig. 2 (a,b) shows the mass spectra for 10 and 5000 pg morphine. Fig. 3a shows intensity versus analyte concentration responses of the peak at *m/z* 286.1431 that can be fitted to $y = 1548x - 10069$ and the R^2 value was 0.997. The response of the signal was controlled by observing a peak from the environment and or chromatography paper at m/z 301 which was identified using MS/MS (LTQ-XL, Thermo Scientific, Bremen, Germany) and exact mass analysis (Orbitrap Exactive) as the sodium adduct ion of dibutyl-phthalate, a commonly used industrial plasticizer.[28,64] By using SPA-nanoESI morphine could directly be detected from the raw urine after depositing the sample on the needle tip or dipping the needle without chromatography paper about 5-10 mm into the morphine-spiked urine solution with the lowest concentration of 1 ppm which has the similar mass spectrum as shown in the Supplementary Fig 2(c). This limit of detection (LOD) is quite useful

for the analysis of real samples as the Federal Workplace Reference LOD of morphine from urine is 2 ppm.[65] By sticking a small piece of chromatography paper on the needle tip during sampling the LOD of morphine could be 500 ppb (500 ng/ml) or lower in raw urine.

Since, the absolute amount of the sample cannot be measured by dipping the needle to the urine-spiked sample solution; we have alternatively deposited these sample solutions on the needle tip and dried for the quantification purposes as mentioned previously. Under optimized experimental conditions, the LOD was about 500 pg or lower for morphine spiked in urine. Supplementary Fig. 2 (c,d) shows the mass spectra for 500 pg and 100 ng morphine in urine. Fig. 3b shows analyte amounts versus intensity responses for urine solution of morphine that can be fitted to $y = 14336x + 19098$, $R^2 = 0.995$. The linearity of this technique was determined by plotting a seven-point calibration curve in the range of 10-5000 pg for pure morphine and a six-point calibration curve in the range of 1-100 ng for morphine-spiked urine using three replicates of each concentration. The intraday precision of this method was expressed by the percentage of relative standard deviation (% RSD) of the five replicate measurements. The RSD for morphine-spiked (1 ng) raw urine was 17.6 %, whereas for the pure sample it was 7.0 %. We speculate that a matrix effect might be the main reason behind the higher RSD value for raw urine compared to the standard sample.[53] Morphine recovery was calculated by depositing 500 nL of urine spiked morphine solution at five different concentration levels of 5 ppm, 10 ppm , 20 ppm, 50 ppm and 100 ppm (absolute amounts 2.5 ng, 5 ng, 10 ng, 25 ng and 50 ng) on the needle tips, and dried at room temperature and six independent measurements were performed for each concentration level by inserting these needles to the 2 μL of methanolic solvent preloaded nano capillaries. We note that salts from the urine cannot be dissolved in methanol completely. Therefore, the

clogging effect of the capillary could be eliminated during the sample measurement. An average of 94.0 % morphine was recovered from raw urine by applying the present technique.

For direct analysis of tablet drugs, samples were stuck by a needle and the sample loaded needle was inserted to a nanoESI capillary loaded with 2 μL of methanol. Supplementary Fig. 3 shows the mass spectra of tablet drugs: (a) erythromycine, (b) ciproxan, and (c) omeprazol. This capability could be value for the real and counterfeit drugs monitoring based on their active pharmaceutical ingredient (API)[66] in a simple and rapid manner. Additionally we have also analyzed proteins in powder form to show the diversity of this technique. The small portion of protein powder (~ micro gram) was picked up by the needle tip and inserted to the nanoESI capillary in which 2 μL of methanol/water (1/1) containing 1% acetic acid was preloaded. Supplementary Fig. 4 demonstrates the mass spectra of proteins: (a) myoglobin, (b) cytochrome c, (c) lysozyme and (d) insulin. All the proteins are detected as major ions.

Matyash et al. reported that accurate profiling of lipidomes relies upon the quantitative and unbiased recovery of the lipid species from analyzed cells, fluids, or tissues and is usually achieved by two-phase extraction with chloroform.[67] In contrast, SPA-nanoESI does not need such extraction procedure. Biofluid could directly be extracted by sticking the needle to the biological tissues. The biofluid attached at the needle tip was then kept at room temperature for three days prior to the analysis to examine the alternative storing and analyzing procedures for thin and dried biofilm. In Fig. 4 (a) for normal kidney tissue, cholesteryl ester and phosphatidylcholines such as $[CE(18 : 14) + Na]^+$, $[PC(34 : 2) + H]^+$, $[PC(34 : 2) + Na]^+$, $[PC(34 : 1) + Na]^+$, $[PC(34 : 1) + K]^+$, $[PC(36 : 2) + Na]^+$, and $[PC(38 : 4) + Na]^+$, as major components and triacylglycerols (TAGs) such as $[TAG(52 : 2) + K]^+$ as minor ions were detected. In contrast,

in cancerous kidney tissues as shown in Fig. 4(b), triacylglycerols such as $[TAG(52 : 3) + Na]^+$, $[TAG(52 : 2) + Na]^+$, $[TAG(54 : 3) + Na]^+$, and $[TAG(54 : 2) + K]^+$, and cholesteryl esters (CE) such as $[CE(22 : 3) + Na]^+$ as major components but negligible phosphatidylcholines such as $[PC(34 : 1) + H]^+$, have been detected. Namely, normal and cancerous kidney tissues are clearly distinguished from dried biomaterials by the present method. Figs. 4 (c-d) show the mass spectra of normal and cancerous kidney tissues without drying on the needle tip. The results obtained before and after drying the biofluids are almost identical (see Figs. 4 (a-d)). As an alternative approach to our previous study, selective detection of lipid and proteins[33] can be occurred by changing solvents from organic to aqueous for the dried biofluids on the needle tip. Supplementary Fig. 5a shows the mass spectrum of normal kidney tissues in which α and β chains of hemoglobin with heme were detected with negligibly weak peaks from the PCs and TAGs when methanol/water (1/1) used as solvent. In contrast, as Supplementary Fig. 5b by using pure methanol different species of phosphatidylcholine and cholesteryl ester such as $[CE(18 : 2) + Na]^+$, $[PC(34 : 1) + H]^+$, $[PC(34 : 2) + H]^+$, $[PC(34 : 2) + Na]^+$, $PC(34 : 1) + Na]^+$, $[PC(36 : 2) + Na]^+$ and$[PC(36 : 1) + Na\ ]^+$, as major components and triacylglycerols (TAGs) such as $[TAG(52 : 2) + Na]^+$, $[TAG(52 : 2) + K]^+$ and $[TAG(54 : 3) + K]^+$ minor ones were detected. One drawback for this technique is the clogging of the tip which sometimes hinders the performance of nanoESI. This drawback can easily be overcome by slight breaking the tip.[68]

Conclusion

The SPA-nanoESI offers following fundamental properties: (a) no notable sample preparation is required during the direct real world dried sample analysis, (b) powder materials can be analyzed directly and (c) analysis time is really short. Since lipidomics research is of importance for in-

depth understanding of pathophysiological events, SPA-nanoESI might be an alternative technique for the quick analysis of lipids from normal, and dried forensic and clinical biomaterials. Furthermore, short analysis time and no special sample preparation make this technique especially attractive. These capabilities are of special attention to the applications in drug monitoring and discovery, clinical diagnostics, and omics field.

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Fig. Captions

Fig. 1: Schematic showing solid probe assisted nanoESI (SPA-nanoESI) using acupuncture needles. (a) Sampling of analytes by sticking an acupuncture needle into powder, tablet drug and tissues with human bloods. (b) Sample picked up by an acupuncture needle is inserted into the solvent-preloaded nanoESI capillary for electrospra**y.**

Fig. 2: Mass spectrum for opium in methanol solution. The mass spectrum is an average of three successive scans. The powder sample was touched by the needle and then the needle was inserted to the solvent loaded nano capillary for elctrospray.

Fig. 3. (a): Signal intensity responses of the protonated peak at *m/z* 286.1431of morphine in methanol by SPA-nanoESI MS: 1548x-10069, R^2 =0.997, recorded from dried pure morphine in water. (b): Signal intensity responses of the protonated peak at *m/z* 286.1431 of morphine in

methanol by SPA-nanoESI MS: 14336x + 19098, R^2 =0.995, recorded from dried morphine with 100% urine on the needle tip.

Fig. 4: (a,b): Positive mode SPA-nanoESI mass spectra for (a) normal and (b) cancerous kidney tissues from biofluids dried on the needle tips. (c,d): Positive mode SPA-nanoESI mass spectra for (c) normal and (d) cancerous kidney tissues from biofluids without drying on the needle tips Solvent: ACN/Methanol/isopropanol (1/1/1).

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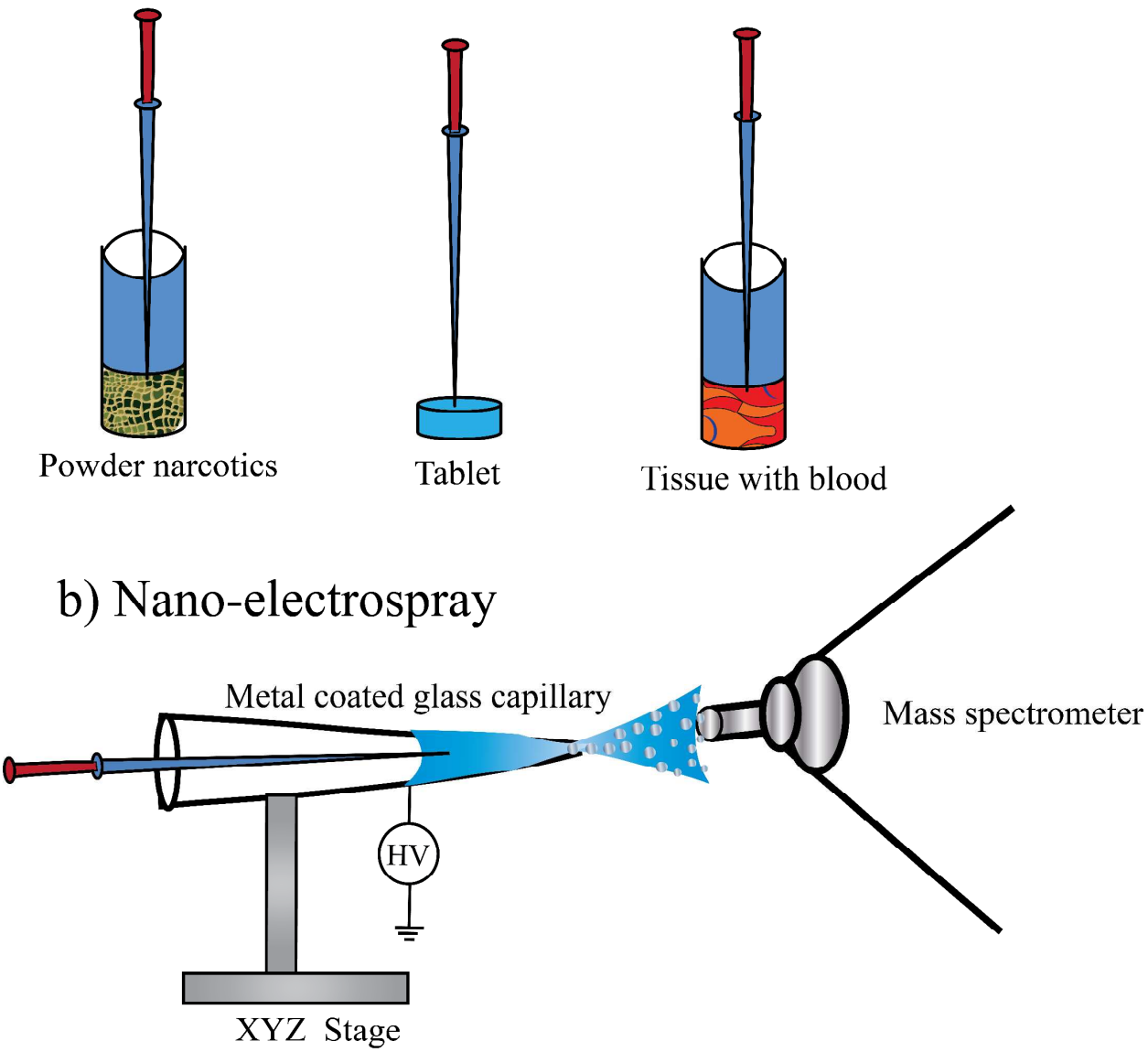


Figure 1

Fig. 1: Schematic showing solid probe assisted nanoESI (SPA-nanoESI) using acupuncture needles. (a) Sampling of analytes by sticking an acupuncture needle into powder, tablet drug and

tissues. (b) Sample picked up by an acupuncture needle is inserted into the solvent-preloaded nanoESI capillary for electrospray.

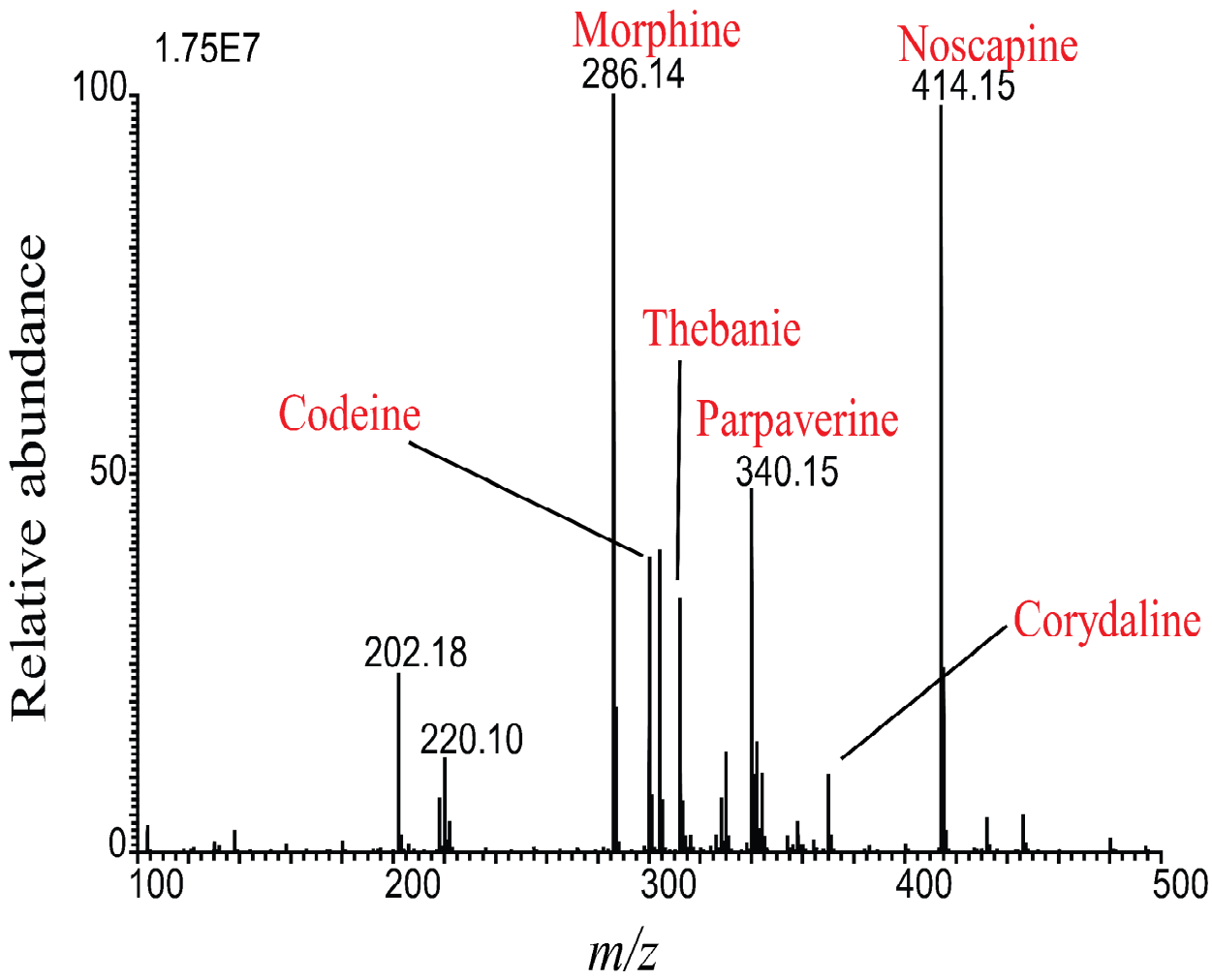


Figure 2

Fig. 2: Mass spectrum for opium in methanol solution. The mass spectrum is an average of three successive scans. The powder sample was touched by the needle and then the needle was inserted to the solvent loaded nano capillary for elctrospray.

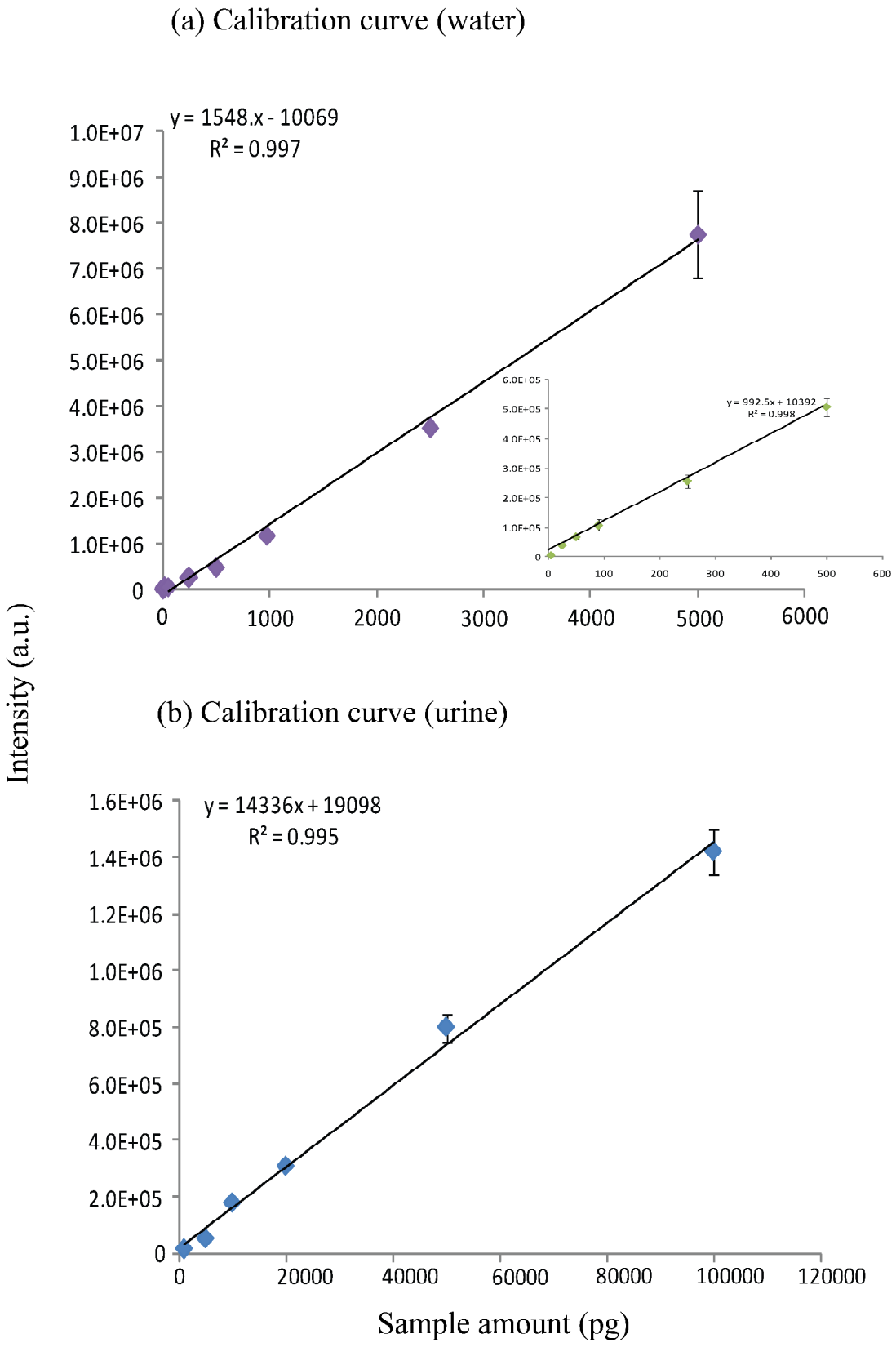

(a) Calibration curve (water)
y = 1548.x - 10069
R² = 0.997
1.0E+07
9.0E+06
8.0E+06
7.0E+06
6.0E+06
5.0E+06
4.0E+06
3.0E+06
2.0E+06
1.0E+06
0
0
1000
2000
3000
4000
5000
6000
y = 992.5x + 10392
R² = 0.998
Intensity (a.u.)
(b) Calibration curve (urine)
y = 14336x + 19098
R² = 0.995
1.6E+06
1.4E+06
1.2E+06
1.0E+06
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0
0
20000
40000
60000
80000
100000
120000
Sample amount (pg)

Figure 3

Fig. 3. (a): Signal intensity responses of the protonated peak at *m/z* 286.1431of morphine in methanol by SPA-nanoESI MS: 1548x-10069, R^2 =0.997, recorded from dried pure morphine in water. (b): Signal intensity responses of the protonated peak at *m/z* 286.1431 of morphine in methanol by SPA-nanoESI MS: 14336x + 19098, R^2 =0.995, recorded from dried morphine with 100% urine on the needle tip.

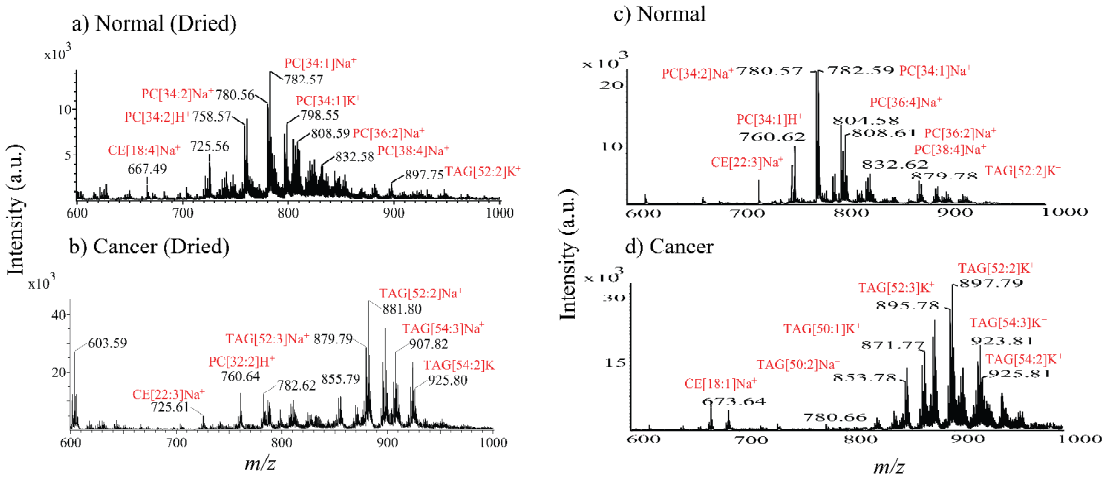
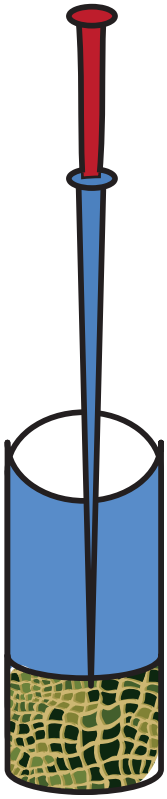
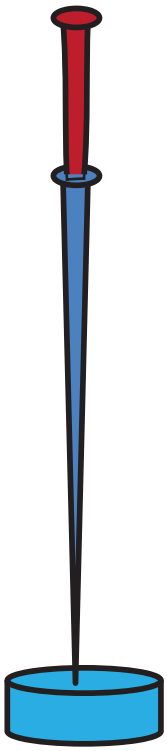


Figure 4

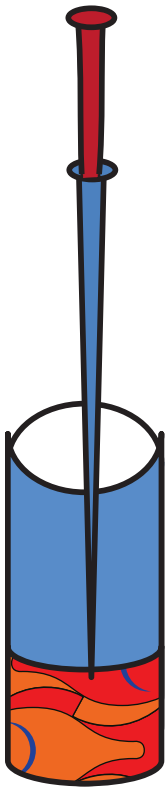
Fig. 4: (a,b): Positive mode SPA-nanoESI mass spectra for (a) normal and (b) cancerous kidney tissues from biofluids dried on the needle tips. (c,d): Positive mode SPA-nanoESI mass spectra for (c) normal and (d) cancerous kidney tissues from biofluids without drying on the needle tips Solvent: ACN/MeOH/isopropanol (1/1/1).



Powder narcotics

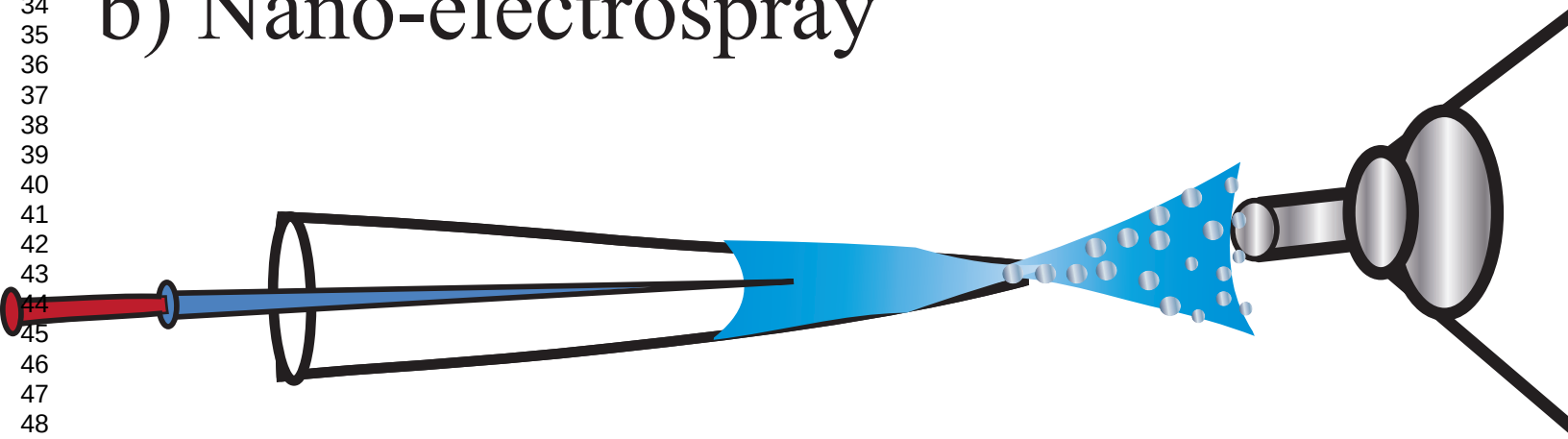


Tablet



Tissue with blood

b) Nano-electrospray



Graphical Abstract