Analysis of Raw Samples by C18 Pipette-tip Electrospray Ionization Mass Spectrometry

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Abstract

A C18 pipette-tip electrospray ionization mass spectrometry technique was developed for rapid analysis of raw samples. In this technique, a C18 pipette tip was employed for rapid purification and enrichment of analytes in raw solutions. The adsorbed analytes were eluted by solvents supplied by a syringe and a syringe pump, and a high voltage was applied onto the syringe needle to induce electrospray ionization at the pipette tip end for mass spectrometric analysis. This technique is simple, easy to assemble, and can be conveniently used for qualitative and quantitative analysis of raw samples with good performance.

1. Introduction

Mass spectrometry (MS) is a powerful tool for qualitative and quantitative analysis of various analytes, and electrospray ionization (ESI) is a commonly used MS technique [1]. ESI-MS analysis of complex samples typically requires labor-intensive sample pretreatment, including extraction, enrichment, chromatographic separation, etc [2-4]. In the past decade, great efforts have been made to enable direct analysis of complex samples by ESI-MS and various ambient ionization techniques have been developed [5-7].

In recent years, our group has devoted to develop new techniques to facilitate ESI-MS analysis [8-14]. Very recently, we developed a pipette-tip ESI-MS technique [14] for direct analysis of raw solid samples. Pipette-tip ESI-MS combines common pipette tips with syringe and syringe pump and allows on-line extraction and ionization of raw solid samples placed inside the pipette tips. We demonstrated that pipette-tip ESI-MS could be conveniently used for rapid analysis of various solid samples, particularly herbal powders, with stable, durable and reproducible signals.

In this study, the pipette tip in the above-mentioned technique was replaced with a C18 pipette tip (Figure 1) and the technique was further developed for analysis of raw samples. Pipette tip-based micro-extraction (TBME) has been well developed for sample desalting, purification and enrichment [2, 15, 16]. C18 pipette tip, i.e., pipette

tip containing C18 sorbent, offers rapid sample preparation for mass spectrometric analysis, and its commercial product, i.e., ZipTip, has been commonly available. Inducing electrospray ionization directly from a pipette tip column has been attempted previously [17, 18], typically involving insertion of a metal wire for connection of the high voltage [17, 18] and use of an additional power supply [18]. As shown in Figure 1, our current technique makes use of the metal syringe needle and solvents for delivery of the high voltage, rendering the device simple and easy to assemble. Moreover, in our technique, continuous and controllable supply of solvents by the syringe pump allows durable and reproducible signals and subsequently reliable quantitation of the analytes. After sample loading and clean-up, analyte molecules bound to C18 bed are eluted with the elution solvent and directly sprayed out for ESI-MS analysis with the application of a high voltage to the syringe needle.

Salts and detergents are commonly present in protein samples [4, 19]. Salts such as NaCl are widely used to mimic the physiological environment of organisms for *in vitro* protein research, while detergents such as sodium dodecyl sulfate (SDS) are commonly employed for protein isolation and solubilization, especially for membrane proteins that are usually of poor solubility [3, 20]. Mass spectrometry is the method of choice for analysis of proteins, however, it is not compatible with salts and detergents. Removal of salts and detergents from protein samples is thus essential prior to mass spectrometric analysis and various methods have been employed [3, 4, 21, 22]. In this

study, C18 pipette-tip ESI-MS was attempted for analysis of protein solutions containing salts and detergents, in an effort to develop simple and rapid approaches for detection of proteins in the presence of salts and detergents.

Drug abuse, especially abuse of psychotropic drugs, is a serious problem worldwide. Abuse of psychotropic drugs could not only harm the health of the abusers, but also bring a lot of social problems. Many kinds of psychotropic drugs are thus prohibited or controlled in most countries in the world. Ketamine is a commonly abused psychotropic drug to induce psychedelic effects to the abusers [23], and norketamine is the major metabolite of ketamine in human body [24, 25]. The abuse of ketamine popularly existed in east and southeast Asia, especially in Singapore and Hong Kong [26], and the involved population has been growing in recent years [27]. Identification of ketamine abusers is critical for drug control, which usually relies on measurements of ketamine residue and norketamine in the abusers' urine, blood, nail and hair [28-31]. Urine is commonly chosen for the measurement due to the relatively high concentrations of ketamine and norketamine and large sample volume available. For identification of ketamine abusers and judicial practice in drug control, a valid and rapid method for quantitative analysis of ketamine and norketamine in urine is essential.

Quantitation of ketamine and norketamine in urine is usually performed using MS combined with gas chromatography (GC) or high-performance liquid chromatography (HPLC) [28, 30, 32-34]. In order to reduce interferences of matrices in the urine samples, extensive sample pretreatments are required before chromatographic separation and MS detection [28, 30, 32-34]. Development of simple, rapid and high-throughput methods are thus highly desirable for the growing analytical demands in beat drugs campaigns. Very recently, direct detection and quantitation of ketamine and norketamine in urine and oral fluid using wooden-tip ESI-MS was successfully developed by our group [35]. The achieved linear range, limit of detection (LOD) and limit of quantitation (LOQ) were 50-5000 ng mL^{-1}, 20 ng mL^{-1} and 50 ng mL^{-1} respectively for quantitation of both ketamine and norketamine in urine [35]. For analysis of various real samples, a linear range with lower concentrations and lower LOD and LOQ may be required [30], and a new method is thus expected. In this study, C18 pipette-tip ESI-MS was examined for quantitation of ketamine and norketamine in urine.

2. Experimental Section

2.1. Materials and Chemicals

C18 pipette tips, i.e., 10 µL ZipTip containing 0.6 µL C18 resin, were purchased from Millipore (USA). The C18 resin was made of silica of 15 µm diameter and 200 Å pore size. The tip (top) I.D. and O.D. are 3.375 mm and 5.8 mm respectively.

Cytochrome *c* from equine heart, myoglobin from equine heart, α-lactalbumin from bovine milk and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Urine was collected from a healthy volunteer. Water was prepared using a Milli-Q system (Millipore Laboratory, USA). All the other solvents were of HPLC grade and purchased from Tedia (Fairfeild, OH, USA).

2.2. Sample Preparation

For the detection of proteins, 5 μM cytochrome c, myoglobin and α-lactalbumin were prepared in the solution of NaCl (1%) or SDS (0.1%). Then the protein solution was mixed with methanol containing 0.2% formic acid (FA) in a volume ratio of 1: 1 for conventional ESI-MS analysis as comparison, or treated with C18 pipette tip for subsequent C18 pipette-tip ESI-MS analysis.

For quantitation of ketamine and norketamine, standard solutions of ketamine or norketamine were prepared with concentrations of 400, 100, 50, 10, 5, 2 μg mL^{-1} in methanol, and internal standard solution of d_4-norketamine was prepared with a concentration of 50 μg mL^{-1} in methanol. Urine samples containing ketamine and norketamine for quantitative analysis were prepared with the followed procedures: 1 mL urine was spiked with 1 μL ketamine and 1 μL norketamine standard solutions of various concentrations (2, 5, 10, 50, 100, 400 μg mL^{-1}), then 1 μL of the internal standard solution was added to each solution. Finally, a set of urine samples

containing ketamine and norketamine with different concentrations (2, 5, 10, 50, 100, 400 ng mL^{-1}) and d_4-norketamine with a fixed concentration (50 ng mL^{-1}) were obtained.

Procedures for using C18 pipette tip for removal of salts, detergents and other interferences were similar to the protocol described in the Millipore C18 ZipTip manual. After the equilibration procedure, typically 10 µL sample was used for sample loading each time, and for quantitation of ketamine and norketamine in urine, this sample loading procedure was repeated six times for enrichment of analytes. After the washing procedure (three times), the C18 pipette tip was combined with the syringe and syringe pump for ESI-MS analysis. Methanol/water/formic acid (50/50/0.1, v/v/v) was used as the extraction and spraying solvent if not specified otherwise.

2.3. Instrumentation and Setup

As shown in Figure 1**,** the blunt point needle (i.d. 410 µm and o.d. 720 µm) of a glass syringe (250 µl, Hamilton) was inserted into the C18 pipette tip. A syringe pump (Harvard Pump 11 Plus, USA) was employed to supply solvents with a flow rate of 5 µL min^{-1}. A high voltage (typically 5.5 kV) was applied to the stainless steel syringe needle and conducted to the pipette tip end through the solvent to induce electrospray ionization. A safety warning was employed to present the severe high voltage hazard to any operator. Mass spectrometric measurements were performed in positive ion mode.

For analysis of the protein solutions, the C18 pipette-tip ESI ion source was coupled with a quadrupole time-of-flight (Q-ToF) mass spectrometer (QStar Pulsar, Applied Biosystems, USA). The protein signals typically lasted for about 30 seconds and the spectra were obtained by accumulating the signal periods. Analysis of the protein solutions with conventional ESI-MS approach was performed on the same mass spectrometer with the equipped ESI ion source. The mass spectrometer was operated with a curtain gas flow of 30 A. U., and the spray voltage (IS), first declustering potential (DP1), focusing potential (FP) and second declustering potential (DP2) were set to optimum values.

For quantitation of ketamine and norketamine in urine, the C18 pipette-tip ESI ion source was coupled with a triple quadruple mass spectrometer (Quattro Ultima, Waters, USA), with the pipette tip end located at a position with a perpendicular distance of 3.0 cm and parallel distance of 1.0 cm to the MS inlet. The capillary voltage, cone voltage and source temperature were set at 3.8 kV, 30 V and 80 °C respectively. The elution and spray solvent used was 80% methanol containing 1.0% FA, and the flow rate is 5 μL min^{-1}. The equipment was performed in positive selected reaction monitoring (SRM) mode. The ion $C_7H_5Cl^+$ at *m/z* 125, the major fragment ion for both ketamine and norketamine, was chosen as the product ion in the SRM scan for the two compounds. The selected reaction $C_{13}H_{17}ClNO^+$ at *m/z* 238

$\rightarrow C_7H_5Cl^+$ at *m/z* 125 for ketamine was monitored with a collision energy of 25 eV and a dwell time of 0.2 s; the selected reaction $C_{12}H_{15}ClNO^+$ at *m/z* 224 $\rightarrow$ $C_7H_5Cl^+$ at *m/z* 125 for norketamine was monitored with a collision energy of 20 eV and the same dwell time of 0.2 s; the selected reaction d_4-$C_{12}H_{15}ClNO^+$ at *m/z* 228 $\rightarrow$ d_4-$C_7H_5Cl^+$ at *m/z* 129 for internal standard d_4-norketamine was monitored with the same conditions as for norketamine. The inter-channel delay time and inter-scan delay time were set at 0.02 s and 0.1 s respectively. The spectra were acquired and processed with the MassLynx^{TM} V4.0 software (Waters, U.S.A).

3. Results and Discussion

3.1 Analysis of Protein Solutions Containing Salts or Detergents

A solution of cytochrome c (5 µM) containing 1% NaCl was analyzed by C18 pipette-tip ESI-MS, in comparison with conventional ESI-MS. As shown in Figure 2A and 2B, only the NaCl clusters and no signals of the protein could be detected in the mass spectrum when the sample was analyzed by conventional ESI-MS. While with C18 pipette-tip ESI-MS, multiply charged ions of cytochrome *c* (+11, +10, +9, +9 and +7) were clearly observed in the spectrum with almost no signal of the salt. Similar results were obtained when another protein solution, 5 µM myoglobin with 1% NaCl, was analyzed. As shown in Figure 3A and 3B, no peaks corresponding to myoglobin were observed in the spectrum obtained with conventional ESI-MS; while with C18 pipette-tip ESI-MS, quality spectrum showing a series of multiply charged

ions of myoglobin (+21, +20, +19, +18, +17, +16, +15, +14, +13, +12 and +11) and the heme (*m/z* 616.2) was obtained. These results demonstrated the capability of C18 pipette-tip ESI-MS for desalting and rapid analysis of protein samples containing salts.

Protein solutions containing detergents were examined as well in this study, and the spectral results are shown in Figure 4. α-Lactalbumin, a common membrane protein, in a solution containing 0.1% SDS was analyzed using both conventional ESI-MS and C18 pipette-tip ESI-MS. Conventional ESI-MS could not detect any protein signals and only the SDS clusters could be obtained. With C18 pipette-tip ESI-MS, the multiply charged ions of α-lactalbumin (*m/z* 1289.9, 1418.8, 1576.3, 1773.2, 2026.4 and 2363.9) could be detected obviously.

The above results indicated that relatively hydrophobic proteins could be retained by nonpolar C18 sorbent, while hydrophilic NaCl and SDS had less affinity with the sorbent and could be readily washed out. The results also demonstrated that C18 pipette-tip ESI-MS could be used for rapid and convenient detection of proteins from solutions containing salts and detergents.

3.2. Quantitation of Ketamine and Norketamine in Urine

We first tested the possibility to repeatedly use one C18 pipette tip for the measurements. SRM mode was employed to eliminate the influence of background and improve the signal sensitivity. The reproducibility using three individual C18 pipette tips or the same C18 pipette tip for three repeat measurements was investigated with a urine sample spiked with 50 ng mL^{-1} ketamine, norketamine and the internal standard, and the obtained SRM chromatograms are shown in Figure 5. For three individual C18 pipette tips, the RSD (relative standard deviation) for the three peak area ratios of ketamine and norketamine to the internal standard was 10.2% and 9.8% respectively, which are acceptable for sample analysis. For the repeat measurements using the same C18 pipette tip, the peak area ratios for ketamine and norketamine were 11.6% and 13.1% respectively, which were in acceptable ranges and indicating no memory effects from the previous measurement. To reduce the experiment costs and make the measurement simpler, one C18 pipette tip was used for the whole measurements. The re-used C18 pipette tip was carefully washed with 200 µL methanol/water/1% FA (50/50/1, v/v/v) each time before the measurement.

Quantitation of ketamine and norketamine in urine was simultaneously performed with C18 pipette-tip ESI-MS, using d_4-norketamine as the internal standard and selected reactions *m/z* 238 → *m/z* 125, *m/z* 224 → *m/z* 125, and *m/z* 228 → *m/z* 125 for the three compounds respectively. The C18 pipette tip allowed rapid sample enrichment and clean-up. A lower concentration range of 2-400 ng mL^{-1} was thus

investigated with this novel technique. The SRM chromatograms for ketamine (*m/z* 238 > 125) and norketamine (*m/z* 224 > 125) in the concentration sequence of 2, 5, 10, 50, 200, 400 ng mL^{-1} and the internal standard d_4-norketamine (*m/z* 228 > 128) with a fixed concentration of 50 ng mL^{-1} are shown in Figure 6. A positive correlation between the peak area and analyte concentration was found both in the analysis of ketamine and norketamine. The peak area of the internal standard for different samples did not vary significantly. As shown in Figure 6, distinct chromatographic peaks with a time window of 40-80 s could be obtained. Since the sample preparation procedure with the C18 pipette tip only needed about 2-3 minutes for each sample, the total time for analysis of each sample could be less than 5 minutes.

For construction of calibration curves, each spiked urine sample was measured for three times, and the mean values of the peak area ratios between analyte and the internal standard and their RSDs were shown in Table 1. The calibration curves for ketamine and norketamine, as shown in Figure 7, were obtained by plotting peak area ratio against the analyte concentration. For both ketamine and norketamine, the calibration curves displayed excellent linear relationship over the examined concentration range of 2-400 ng mL^{-1}. The coefficient R^2 was very close to 1 for both ketamine (R^2 =0.9999) and norketamine (R^2 = 0.9998). This linear range covered the concentration ranges of ketamine and norketamine in common drug abusers' urine [30], indicating the applicability of the present technique in real applications. This

achieved linear range was comparable to the conventional HPLC-MS and GC-MS in the previous reports [28, 29, 32-35]. The RSDs for analysis of each sample were listed in Table 1, which were acceptable for the measurements.

The precision and accuracy for quantitation of ketamine and norketamine in urine with C18 pipette-tip ESI-MS were investigated as well (Table 2). A low concentration at 20 ng mL^{-1} and a high concentration at 200 ng mL^{-1} were measured. For ketamine, the precisions were determined to be 13% and 5%, and the accuracies were determined to be 94% and 90%, at the two concentrations respectively. For norketamine, the precisions were 5% and 7%, and accuracies were 98% and 89%, at the two concentrations respectively. These results were comparable to those obtained with conventional methods [28-30, 32-35].

The LOD and LOQ of this method were determined by comparing the peak height of the analyte with the average height of the noises after the analyte peak. The LOD and LOQ were defined as the concentration of the analyte when the signal of the analyte is three and ten times of the signal of the noises respectively. The LOD and LOQ for the present technique were determined to be 0.3 ng mL^{-1} and 0.5 ng mL^{-1}, respectively, for ketamine and 0.8 ng mL^{-1} and 1.0 ng mL^{-1}, respectively, for norketamine. These LOD and LOQ were good enough for analysis of real samples.

4. Conclusions

By combining C18 pipette tip, a device for rapid purification of samples, with syringe and syringe pump, a C18 pipette-tip ESI-MS technique was developed. In this technique, analyte in raw samples was concentrated and purified by C18 pipette tip rapidly and directly eluted to generate electrospray ionization for mass spectrometric analysis. Because of the enrichment of analyte with C18 pipette tip, the obtained mass spectra showed better signal sensitivity. Using this novel technique, rapid detection of proteins from solutions containing salts such as sodium chloride or detergents such as SDS, and quantitation of ketamine and norketamine in human urine with desired analytical performance, were successfully achieved. The linear range, precision, accuracy, LOD and LOQ for both ketamine and norketamine quantitation with this method were well acceptable for analysis of real samples. This method is very simple, easy to operate, cost-effective (the C18 pipette tip could be reused), and thus very useful for rapid analysis of raw samples. The C18 sorbent can also be replaced with other chromatographic materials and be used for other analytical purposes.

Acknowledgments

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Table 1. Data for quantitation of ketamine and norketamine.

Concentration (ng mL^{-1})	Ketamine		Norketamine	
	Peak area ratio (analyte/IS)	**RSD (n =3, %)**	**Peak area ratio (analyte/IS)**	**RSD (n =3, %)**
2	0.1066	3.05	0.0978	11.13
5	0.1755	3.02	0.1804	7.60
10	0.3070	3.02	0.4966	4.47
50	1.6935	5.04	2.1889	8.50
100	3.1496	5.82	4.0405	6.15
400	12.8589	5.43	16.7950	8.03

Table 2. Precision and accuracy for quantitation of ketamine and norketamine in urine with C18 pipette-tip ESI-MS.

	Ketamine			Norketamine		
Spiked Concentration ($ng\ mL^{-1}$)	**Measured Concentration ($ng\ mL^{-1}$)**	**Precision (n=6)**	**Accuracy (n=6)**	**Measured Concentration ($ng\ mL^{-1}$)**	**Precision (n=6)**	**Accuracy (n=6)**
20	18.9	13%	94%	19.6	5%	98%
200	179.2	5%	90%	177.1	7%	89%

Figure captions

Figure 1. The schematic diagram of C18 pipette-tip ESI-MS.

Figure 2. Spectra obtained by analysis of cytochrome *c* (5 μM, 1% NaCl) with conventional ESI-MS **(A)** and C18 pipette-tip ESI-MS (after rapid clean-up) **(B).**

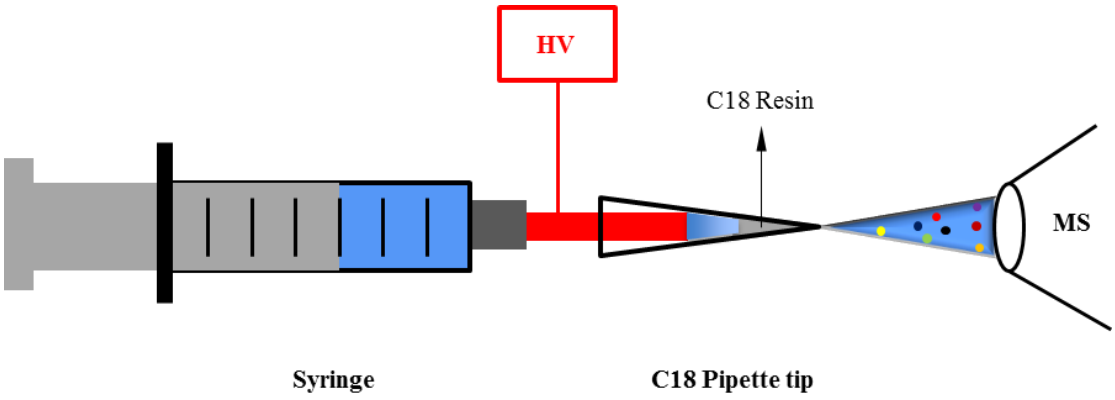
Figure 3. Spectra obtained by analysis of myoglobin (5 μM, 1% NaCl) with conventional ESI-MS **(A)** and C18 pipette-tip ESI-MS (after rapid clean-up) **(B)**.

Figure 4. Spectra obtained by analysis of α-lactalbumin (5 μM, 0.1% SDS) with conventional ESI-MS **(A)** and C18 pipette-tip ESI-MS (after rapid clean-up) **(B)**.

Figure 5. The SRM chromatograms of three measurements using three individual C18 pipette tips (left) and the same C18 pipette tip (right).

Figure 6. The SRM chromatograms for simultaneous detection of ketamine (*m/z* 238 > 125, 2-400 ng mL^{-1}), norketamine (*m/z* 224 > 125, 2-400 ng mL^{-1}) and the internal standard d_4-norketamine (*m/z* 228 > 129, 50 ng mL^{-1}).

Figure 7. Calibration curves for ketamine **(A)** and norketamine **(B)**.



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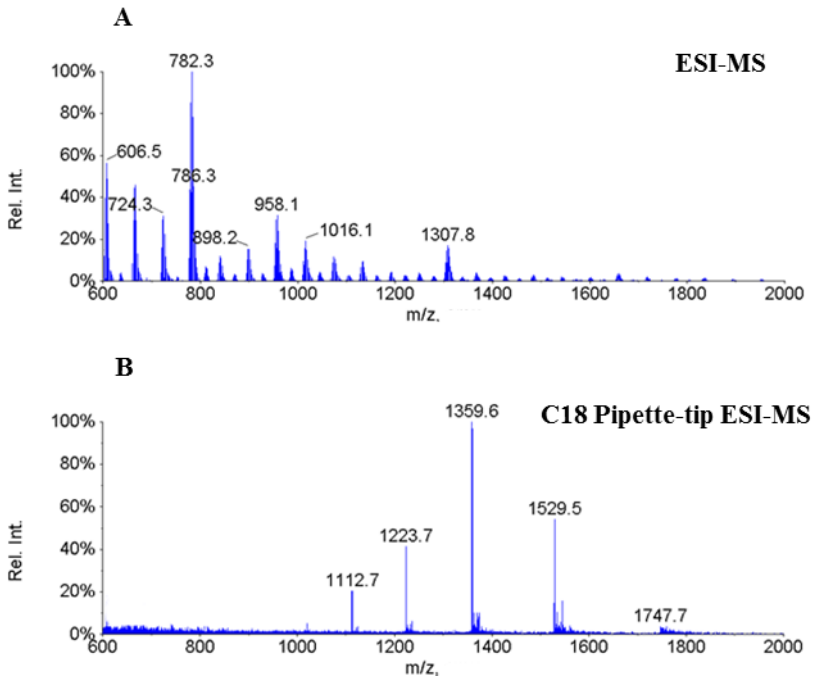
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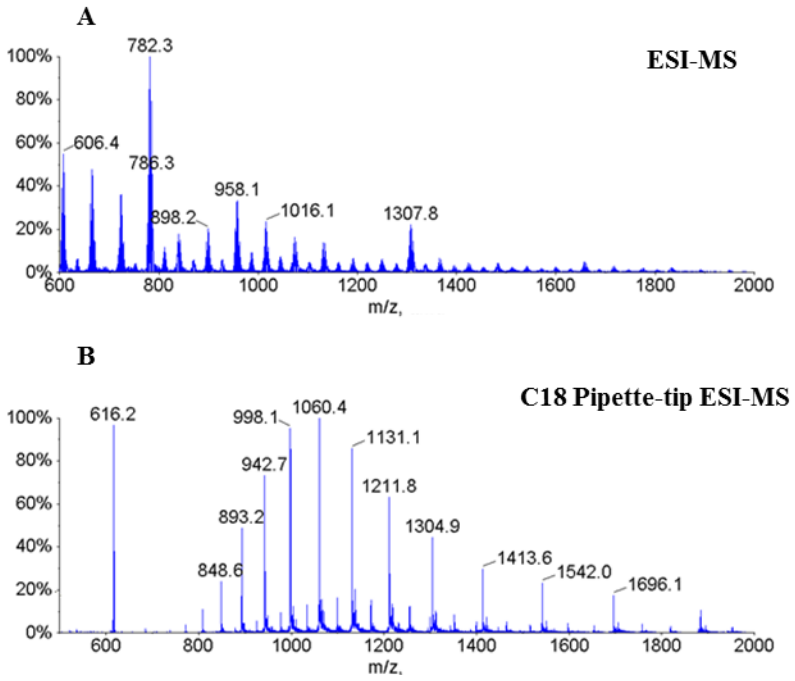
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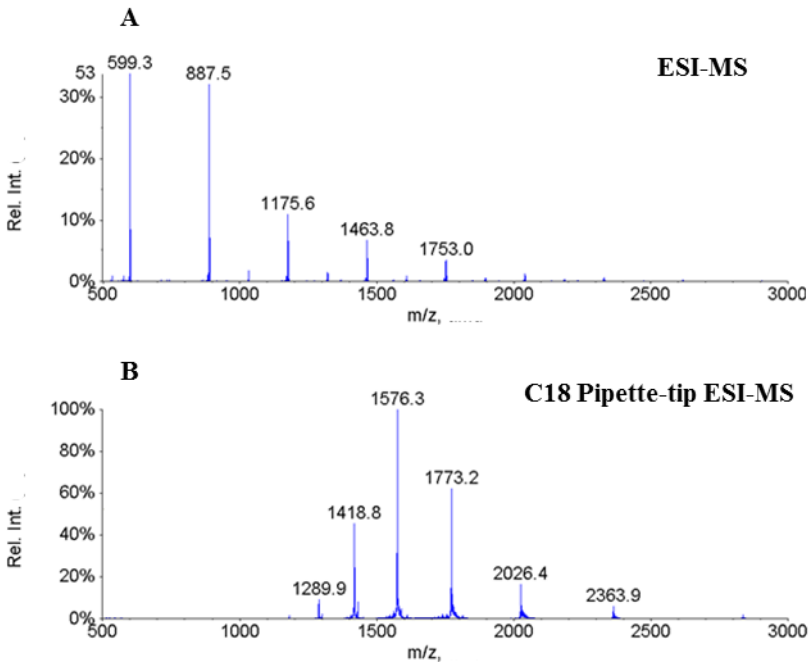
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0%
Rel. Int.
500
1000
1500
2000
2500
3000
m/z,

Figure 4.

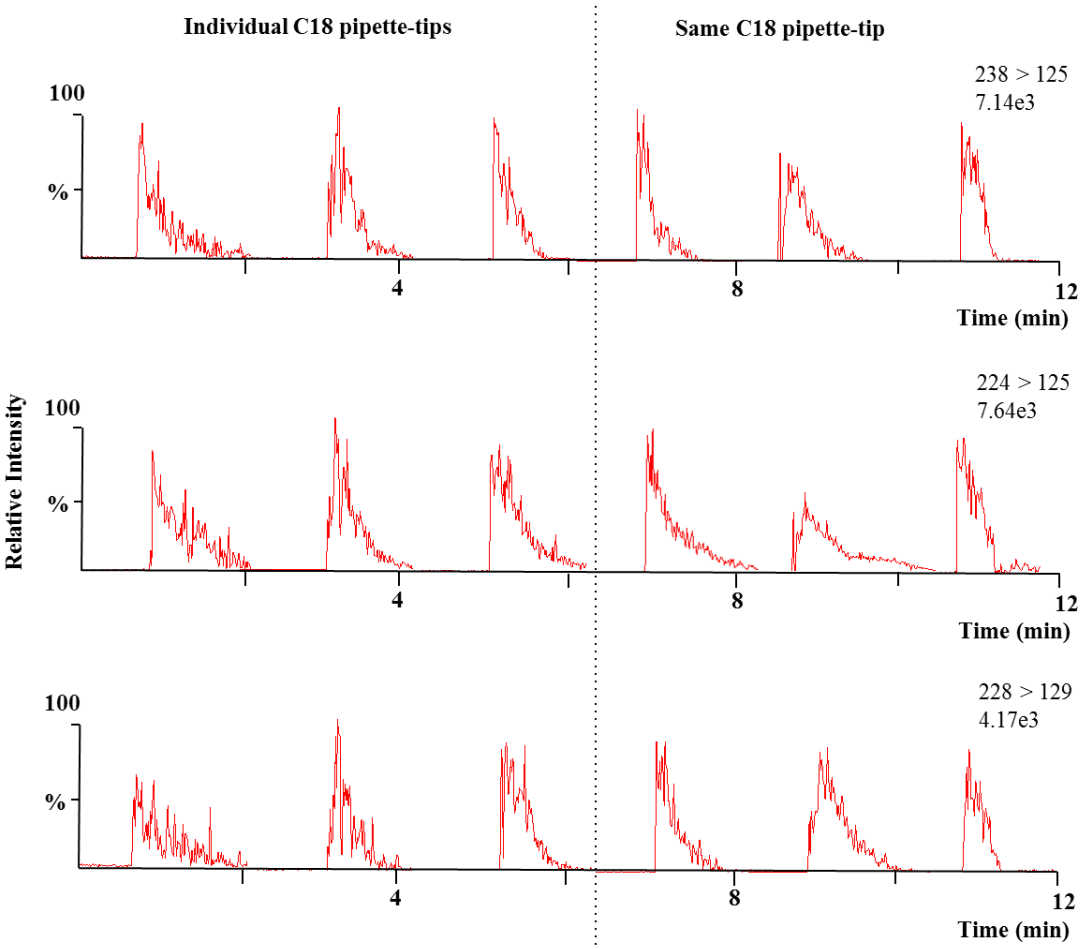
Individual C18 pipette-tips
Same C18 pipette-tip
238 > 125
7.14e3
224 > 125
7.64e3
228 > 129
4.17e3
100
%
4
8
12
Time (min)
Relative Intensity

Figure 5.

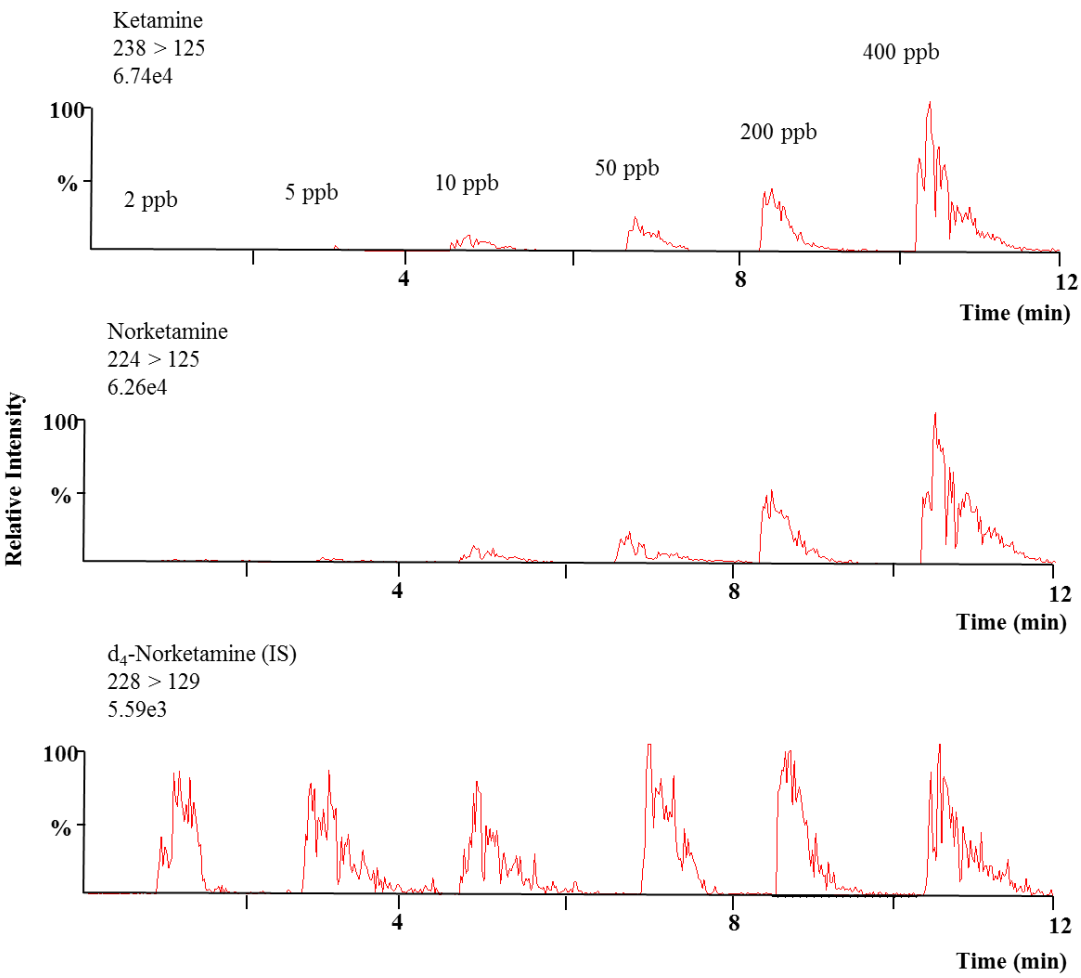
Ketamine
238 > 125
6.74e4
100
%
2 ppb
5 ppb
10 ppb
50 ppb
200 ppb
400 ppb
4
8
12
Time (min)
Norketamine
224 > 125
6.26e4
100
%
4
8
12
Time (min)
Relative Intensity
d4-Norketamine (IS)
228 > 129
5.59e3
100
%
4
8
12
Time (min)

Figure 6.

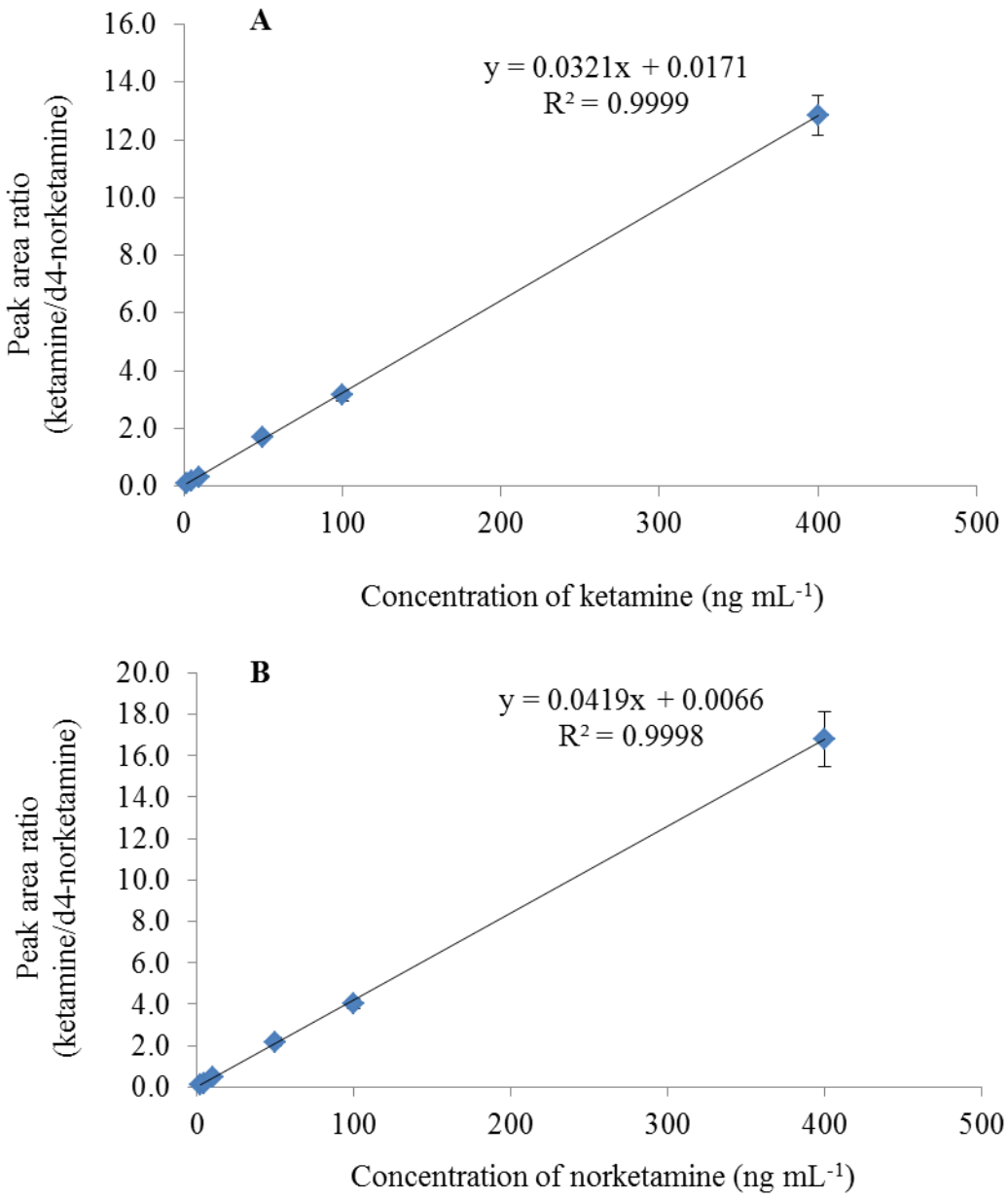
A
y = 0.0321x + 0.0171
R² = 0.9999
Peak area ratio
(ketamine/d4-norketamine)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
100
200
300
400
500
Concentration of ketamine (ng mL-1)
B
y = 0.0419x + 0.0066
R² = 0.9998
Peak area ratio
(ketamine/d4-norketamine)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
100
200
300
400
500
Concentration of norketamine (ng mL-1)

Figure 7.