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Thin Layer Chromatography Coupled with Electrospray Ionization Mass Spectrometry for Direct Analysis of Raw Samples

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Abstract

Conventional mass spectrometric analysis of raw samples commonly require sample pretreatment and chromatographic separation using high performance liquid chromatography or gas chromatography, which could be time-consuming and laborious. In this study, thin layer chromatography (TLC) coupled with electrospray ionization mass spectrometry (ESI-MS) was developed for direct analysis of raw samples. The sorbent material of the TLC plate was found to be able to retain the interfering compounds and allow interested analytes to be extracted, ionized and detected by ESI-MS with much reduced matrix interference. Our results showed that this method could be effectively applied in direct analysis of samples containing common interfering compounds, e.g., salts and detergents, and rapid detection and quantitation of target analytes in raw samples. Offline and online separation and detection of different components in mixture samples, e.g., plant extracts, using TLC-ESI-MS were also demonstrated. Overall, this study revealed that TLC-ESI-MS could be a simple, rapid and efficient method for analysis of raw samples.

Keywords: Mass spectrometry, Electrospray ionization, Thin layer chromatography, Matrix interference, Direct analysis

1. Introduction

Mass spectrometry (MS) is an important analytical technique in chemical and biological fields because of its high speed, sensitivity and specificity. However, analysis of raw samples by MS usually requires extensive sample pretreatment and chromatographic separation using high performance liquid chromatography (HPLC) or gas chromatography (GC), which could be laborious and time-consuming. In the past decades, development of sampling and ionization methods for direct analysis of raw samples has been an important research area in MS. For instance, the development of various ambient ionization techniques, e.g., direct analysis in real time (DART) [1], desorption electrospray ionization (DESI) [2] and electrospay-assisted laser desorption ionization (ELDI) [3], which are characterized by that samples are ionized under atmospheric pressure and no or only little sample preparation is involved, significantly reduces the time and cost required for analysis of raw samples [4-6]. In addition, the techniques of solid-substrate ESI, in which sample solution is loaded and ionized on the open surface of a solid substrate, e.g., paper [7], wooden tips [8, 9], aluminium foil [10], and metal needles [11-13], were also developed to facilitate mass spectrometric analysis of complex samples by avoiding the problem of clogging potentially encountered in conventional capillary-based ESI, allowing more convenient sampling and reducing matrix interferences [11, 14, 15]. The techniques of ESI with paper (paper spray) and wooden tips (WT-ESI) were successfully applied in direct detection of analytes in raw samples, e.g., blood, urine and oral fluid samples, and the separation effects of filter paper and wooden-tip surface were believed to be related to their capabilities for direct sample analysis [8, 9, 16-19].

Thin layer chromatography (TLC) is a simple, rapid and more efficient separation method, and thus coupling of TLC with MS has good potential for direct analysis of raw samples. Coupling of TLC with MS has attracted much attention [20, 21], and various ionization methods, including fast atom bombardment (FAB) [22, 23], matrix-assisted laser desorption/ionization (MALDI) [24], and ambient ionization methods such as liquid extraction junction [25-27], desorption electrospray ionization (DESI) [28, 29], direct analysis in real time (DART) [30-32], easy ambient sonic-spray ionization (EASI) [33] and electrospray laser desorption ionization (ELDI) [34], has been employed for the coupling. In addition, direct coupling of TLC with ESI, in which separated sample spots were cut out for directly ESI or samples were separated online and then directly sprayed out, was also demonstrated [35-37]. However, the major purpuses of these coupling studies were to facilitate identification of the separated TLC spots by MS or online separation and detection of compounds in simple mixtures. In this study, we mainly aimed to employ TLC plate as a solid substrate medium to reduce matrix interference and enhance detection of target analytes in raw samples by ESI-MS. Separation and identification of components in mixture samples were also investigated in this study. Our results indicated that TLC coupled with ESI-MS (TLC-ESI-MS) could be a simple, rapid and efficient method for detection, including quantitative measurements, of analytes in raw samples.

2. Material and methods

2.1 Materials

Acetone, ligroin, sodium chloride (NaCl), sodium dodecyl sulfonate (SDS), octly β-D-glucopyranside (OG), myoglobin and lysozyme were purchased from Sigma (St. Louis, MO). Gramincidin D (from *bacillus breris*) was purchased from International Lab (USA). Ketamine was purchased from Alfasan (Woerden, Holland). Methanol, acetonitrile and chloroform were purchased from Tedia (Fairfield, OH). Ketamine-D4 was purchased from Cerilliant (Round Rock, Texas). Petroleum was purchased from Fisher Scientific (Pittsburgh, Pa.). Formic acid was purchased from Fluka (Buchs, Germany). Ethyl acetate was purchased from Acros (Fairlawn, NJ). Ginsenoside Rc was purchased from Shanghai Tauto Biotech (Shanghai, China). The urine sample was collected from a healthy male volunteer. Herbal medicines Fruit of *Schisandra sphenanthera* (FSS) and Fruit of *Schisandra chinensis* (FSC) were purchased from pharmacy stores in Hong Kong. Fresh spinach leaves were purchased from a supermarket in Hong Kong. Extracts of spinach leaves, FSS and FSC, were prepared as described previously [38]. TLC plates (Model: Alugram Sil G/UV254) were purchased from Macherey-Nagel (Düren, Germany).

2.2 Thin layer chromatography-Electrospray Ionization Mass spectrometry (TLC-ESI-MS)

2.2.1 TLC plates

The TLC plates used in this study have an aluminum base coated with silica gel stationary phase particles. Prior to sample loading, the TLC plates were cut into a dimension of 20 ×15 mm (L × W) and one end of each plate was cut into V-shape with a tip angle of ~ 60^{o}.

2.2.2 TLC-ESI-MS coupling

TLC-ESI-MS experiments were performed on a quadrupole-time-of-flight mass spectrometer (Q-TOF2, Waters-Micromass) or a triple quadrupole mass spectrometer (Quattro Ultima, Waters-Micromass). The experimental setup was similar to the direct coupling method reported previously [35]. Briefly, a TLC plate, which has an aluminum base for conduction of electric current, was cut into triangle shape and positioned ~ 8 mm from the inlet of the mass spectrometer with a clip connected with the high voltage supply of the mass spectrometer (Figure 1). During data acquisition, the capillary voltage and cone voltage were set at 3.5 kV and 30 V, respectively. The ion source temperature was set at 80 ^{o}C and all desolvation gases were turned off. Nano-ESI experiments were carried as similar to our previous study [9].

2.3 Sampling methods

2.3.1 Sampling for analysis of samples with interfering contaminants and raw urine

Sample solution, typically 2 µl, was applied onto the TLC plate at a position ~ 8.0 mm away from the sharp tip end. The sample solution appeared to dry quickly after the sample loading. The TLC plate with loaded sample was first mounted in front of the MS inlet and high voltage was applied to the plate. Ten microliter of elution solvent was then applied around the sample spot position for extraction of the sample. Electrospray could be generated when the elution solvent, i.e., methanol for small organic molecules and ACN/H_2O 50:50 with 0.5% formic acid for peptides and proteins, reached the tip end of the plate.

2.3.2 Analysis of plant extracts: Offline and online samplings

For offline sampling, the plant extract was pre-separated on a TLC plate and the sample spots were located with an UV lamp (Spectronics Corp, NY). The plate regions with sample spots of interest were cut into a triangle shape with a size covering the entire sample spot, typically with ~6 mm base and tip angel of ~ 45^{o}, which were then mounted in front of the MS inlet with a clip connected with high voltage. Ten microliter of elution solvent, i.e., methanol, was added onto the plate for elution of the sample for MS analysis.

For online sampling, sample solution was loaded ~ 20 mm from the tip end of the TLC plate and stood until dryness. The plate with sample was then mounted in front of the MS inlet and connected with high voltage. Ten microliters of developing solvent (acetone/ligroin, 1/9, v/v) was applied onto the sample spot for several repeated cycles until the pigments were seen to travel and separate along the TLC plate to the tip end.

3. Results and discussion

3.1 Analysis of samples containing salts and detergents

Salts and detergents are common interference compounds that can significantly suppress signals of analytes in ESI-MS. The capability of TLC-ESI-MS in analysis of samples containing salts and detergents was investigated in this study. First, methanoic solutions of 1 μM of ginsenoside Rc (GRc), an active component of ginseng herbs [39], containing different concentrations of NaCl, were analyzed with TLC-ESI-MS. For samples containing 1 to 100

mM of NaCl, sodiated ion of GRc at *m/z* 1101 was predominately observed and no interfering ion signal from cluster ions of NaCl was detected (Figures 2a-c). When the concentration of NaCl was increased to as high as 1 M, the interfering signals from NaCl cluster ions were observed, yet the sodiated ion of GRc remained the major detected peak (Figure 2d). As a comparison, when NaCl-containing samples were analyzed by nano-ESI, which has a higher salt tolerance than normal ESI, interfering signals of NaCl cluster ions were detected even at low salt contents, i.e., 1 mM, and completely masked the signals of GRc at 1M NaCl (Figures 2f-i). Control experiments were performed to confirm that the reduced salt interference was not due to dilution of salts by the elution solvent. An aliquot of 2 μL solution containing 1 μM GRc and 2 M NaCl was diluted with 10 μL methanol and analyzed with nano-ESI. In parallel, TLC-ESI-MS analysis with the same dilution conditions, i.e., applying 2 μL of the same solution onto a TLC plate followed by addition of 10 μL of methanol as the elution solvent, was carried out and the two results were compared. The results showed that the ion signals for GRc could be clearly detected with TLC-ESI (Figures 2e), while no ion signal of GRc and only significant ion signals of the salt clusters could be detected with nano-ESI (Figure 2j). These observations revealed that the salt interference could be significantly reduced with the present TLC-ESI-MS method, but not by simple dilution of the salt content. These results indicated that the present TLC-ESI appraoch allowed rapid detection of analytes in high salt content with reduced salt interference, which could be achieved by retention of salts by the polar silica gel stationary phase and selective elution of the more nonpolar analyte for MS detection.

The salt tolerance of the present TLC-ESI-MS method for analysis of proteins with high salt contents was also investigated with horse heart myoglobin. As shown in Figure 3a, the mass spectrum obtained with TLC-ESI-MS showed predominant protein ion signals with no interfering signal from NaCl ion clusters. When the same sample was analyzed with nano-ESI, however, the interfering signals from NaCl cluster ions were predominately detected (Figure 3b). These results further demonstrated that TLC-ESI-MS could be effectively applied in analysis of samples containing high contents of salts.

Detergents are commonly used to improve the solubilities of membrane peptides and proteins, and analysis of membrane peptides and proteins has been a challenging problem in analytical chemistry and protein studies [40, 41]. In this study, the TLC-ESI-MS method was applied to analyze membrane peptide samples containing detergents. The mass spectrum obtained for 50 μM of gramicidin D, a membrane peptide, containing 5 % (w/w) of octyl β-D-glucopyranside (OG) is shown in Figure 4a. The doubly charged sodiated ion ($[M + 2Na]^{2+}$) of gramicidin D could be unambiguously detected with the TLC-ESI-MS approach, although the detergent signals were also observed. However, no ion signal of gramicidin D and only predominate detergent signals were detected when the same sample was analyzed with nano-ESI (Figure 4b). Similar observations were obtained for a protein sample, lysozyme, containing SDS, a commonly interfering detergent in proteomics studies. The analyte of interest could be clearly detected with the TLC-ESI-MS approach, but not with nano-ESI (Figures 4c & d). These data reveal the applicability of the present TLC-ESI-MS method in analysis of samples containing detergents.

3.2 Detection and quantitation of analytes in raw urine

Detection and quantitation of analytes in raw biological fluids, e.g., urine, usually requires sample pre-treatment and HPLC or GC separation. In this study, the capability of TLC-ESI-MS in rapid detection and quantitation of ketamine, a commonly recreational drug, in raw urine was investigated. The quantitation settings were similar to what we used previously for wooden-tip ESI [8]. Upon loading of the raw urine samples spiked with ketamine (0.1, 0.2, 1, 2, 5 and 10 ppm) and ketamine-d4 internal standard (1 ppm) onto the TLC plate and monitoring the analyte and internal standard by selected reaction monitoring (SRM), distinctive SRM signals of ketamine (*m/z* 238> 125) (Figure 5a) and ketamine-d4 (*m/z* 242 > 129) (Figure 5b) could be obtained. The peak intensity (peak height) ratio of ketamine and ketamine-d4 internal standard ($I_{ketamine}/I_{ketamine\text{-}d4}$) exhibited a good linear relationship over the concentration range of 0.1 – 10 ppm (Figure 5c), which was comparable to the results obtained by wooden-tip ESI and within the quantity range commonly found in drug abusers [8]. The limit-of-detection (LOD) and limit of-quantitation (LOQ) were determined by comparing the $I_{ketamine}/I_{ketamine\text{-}d4}$ of the spiked samples, i.e., samples spiked with both analyte and internal standard, with that of the blank sample, i.e., the sample spiked with internal standard only $[(I_{ketamine}/I_{ketamine\text{-}d4})_{spiked}/(I_{ketamine}/I_{ketamine\text{-}d4})_{blank}]$. The LOD and LOQ are defined as the quantity of analyte that could achieve a $[(I_{ketamine}/I_{ketamine\text{-}d4})_{spiked}/(I_{ketamine}/I_{ketamine\text{-}d4})_{blank}]$ of three and ten, respectively. With the present TLC-ESI-MS method, the LOD and LOQ were determined as 30 ppb and 90 ppb, respectively, which are acceptable for real applications. Multiple analysis of a urine sample

spiked with 100 ppb of ketamine and 1 ppm of ketamine-d4 internal standard (n = 9) showed a relative standard deviation (RSD) of 9.5 %, indicating an acceptable level of reproducibility. The above results indicated that TLC-ESI-MS could be used for selective and quantitative detection of analytes in raw samples.

3.3 Characterization of different components in mixture samples

Two sampling approaches, offline sampling and online sampling, were applied for rapid analysis of mixture samples by TLC-ESI-MS. The offline sampling approach was applied for differentiation of Fruit of *Schisandra sphenanthera* (FSS) and Fruit of *Schisandra chinensis* (FSC), two common Traditional Chinese Medicines distributed in southern China and northen China, respectively [42]. The FSC is considered to be of higher quality than FSS [42]. The methanolic extracts of the two herbs were separated on a TLC plate and the sample spots were identified under an UV lamp. The TLC profile of FSC showed five major spots and that of FSS showed three major spots (Figure 6a). By cutting out each spot for TLC-ESI-MS analysis, ion signals for various lignans could be clearly detected. Our results showed that gomisin A and schizandrin B were detected only for FSC, and other lignans, including schizandrin, schisantherin A and schisandrin A, were detected for both FSC and FSS (Figures 6b-f). These results were similar to the previous report [42] and allowed differentiation of the two closely related herbal species. These data indicated the capability of the TLC-ESI-MS method in rapid identification of chemical components in plant extracts with high reliability.

An online sampling method, which does not require separation of samples in an external

container, for analysis of mixture samples was also investigated. An extract of spinach was analyzed with the online TLC-ESI-MS approach and the results are shown in Figure 7. When the developing solvent was loaded onto the sample spot, a yellow-orange component was first separated out, and a predominate peak corresponding to carotene ($[M+H]^+$: *m/z* 537), a major pigment in spinach [43], could be detected (Figure 7a). Following the separation and detection of the yellow carotene component, a grey-green component appeared to separate out and a major peak corresponding to pheophytin A ($[M+H]^+$: *m/z* 871), another well known pigment in spinach [43], was observed (Figure 7b). These data indicated the potential of TLC-ESI-MS for rapid online separation and detection of components in mixture samples. However, care should be taken to allow solvent migration and component separation compatible with spray rate and ESI-MS detection.

4. Conclusion

This study presented a TLC-ESI-MS method for direct analysis of raw samples. Our results showed that the TLC plate could serve as a medium for absorbing interfering substances, allowing target analytes to be detected with reduced matrix interference. This mode of separation of analytes and interfering substances avoided the need of laborious and time-consuming sample pretreatment and HPLC or GC separation in analysis of complex samples. This method was shown to be effective in direct analysis of samples with interfering substances, e.g., salts and detergents, and rapid detection and quantitation of target analyte in raw biological fluids. In addition, the TLC-ESI-MS method was also demonstrated to be an effective method for rapid identification of different components in mixture samples. In view

of the capability in rapid analysis of raw samples demonstrated here, the availability of TLC plates with various sorbent materials and the features of simplicity, economy and easy operation, TLC-ESI-MS could be a decent analytical technique potentially extended to a widely range of chemical and biological applications.

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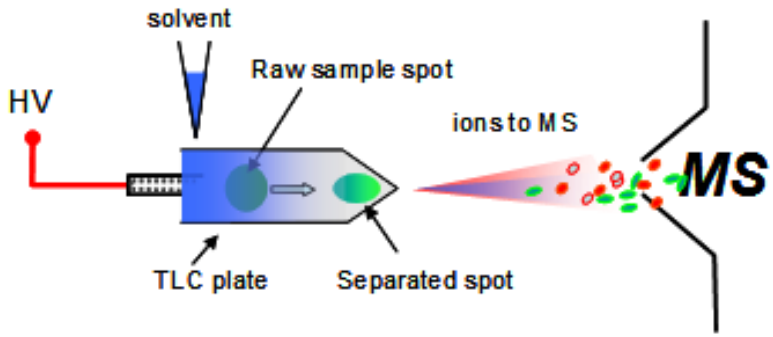
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Raw sample spot
HV
ions to MS
MS
TLC plate
Separated spot

Figure 1

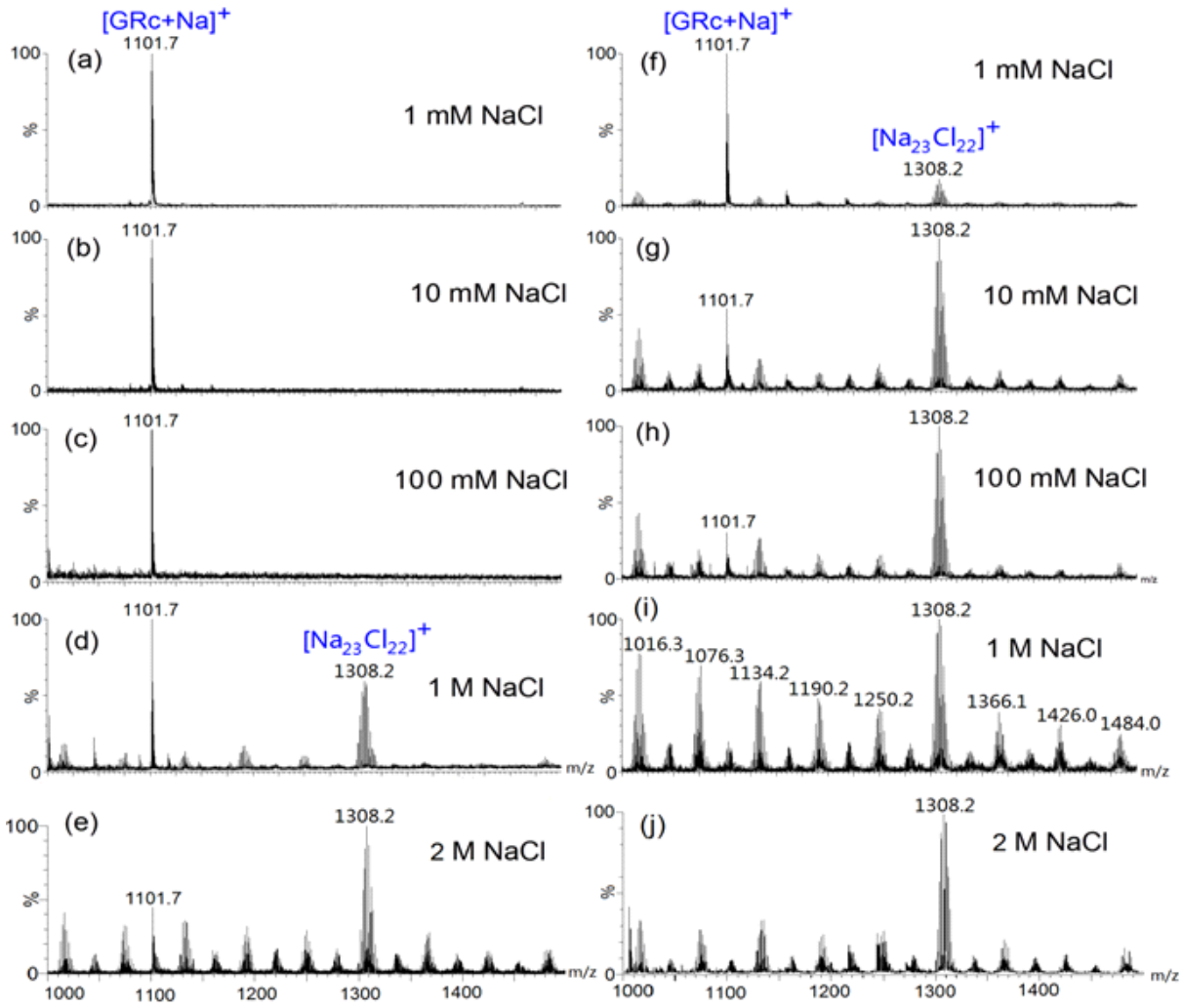
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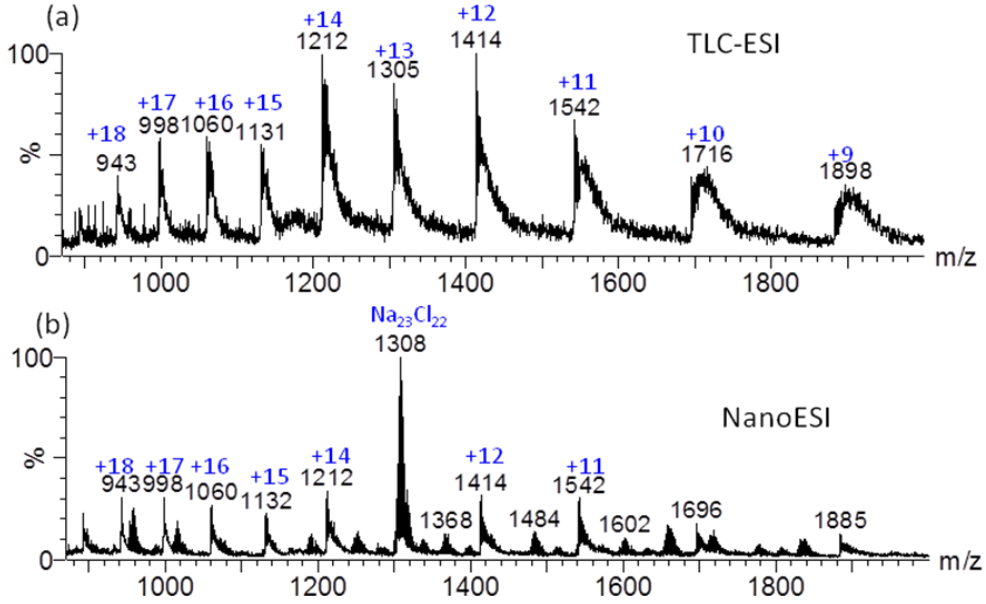
Figure 2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1800
m/z
(b)
100
%
0
Na23Cl22
1308
+18
943
+17
998
+16
1060
+15
1132
+14
1212
+12
1414
+11
1542
1368
1484
1602
1696
1885
NanoESI
1000
1200
1400
1600
1800
m/z

Figure 3

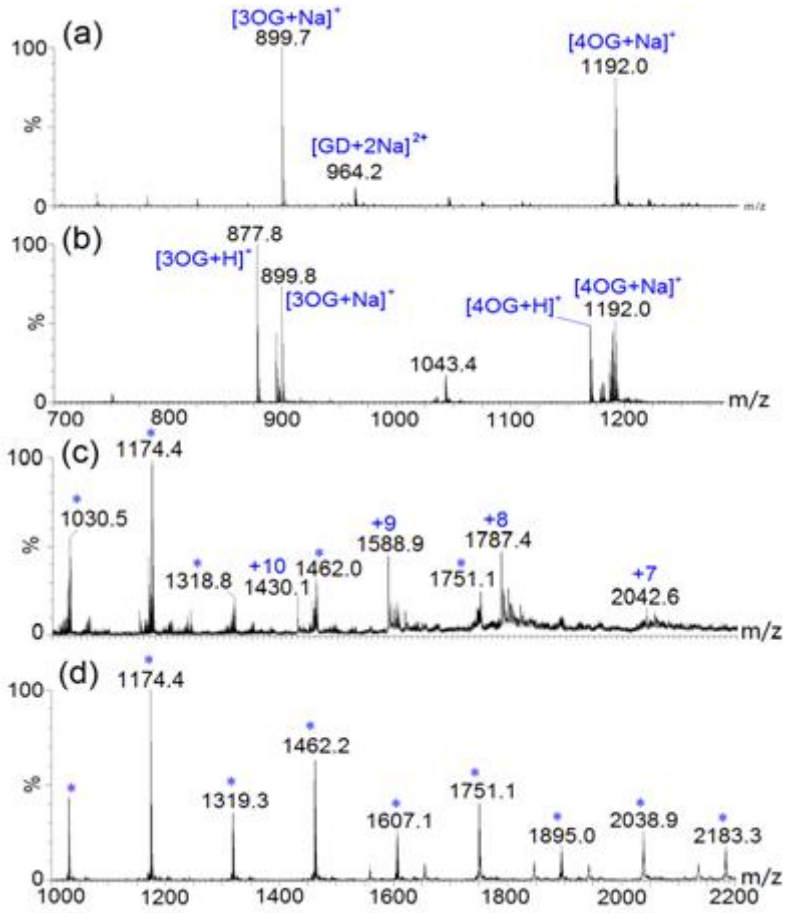
(a)
[3OG+Na]+
899.7
[4OG+Na]+
1192.0
[GD+2Na]2+
964.2
(b)
877.8
[3OG+H]+
899.8
[3OG+Na]+
[4OG+H]+
[4OG+Na]+
1192.0
1043.4
m/z
(c)
1174.4
1030.5
+9
1588.9
+8
1787.4
1318.8
+10
1430.1
1462.0
1751.1
+7
2042.6
m/z
(d)
1174.4
1462.2
1319.3
1751.1
1607.1
1895.0
2038.9
2183.3
m/z

Figure 4

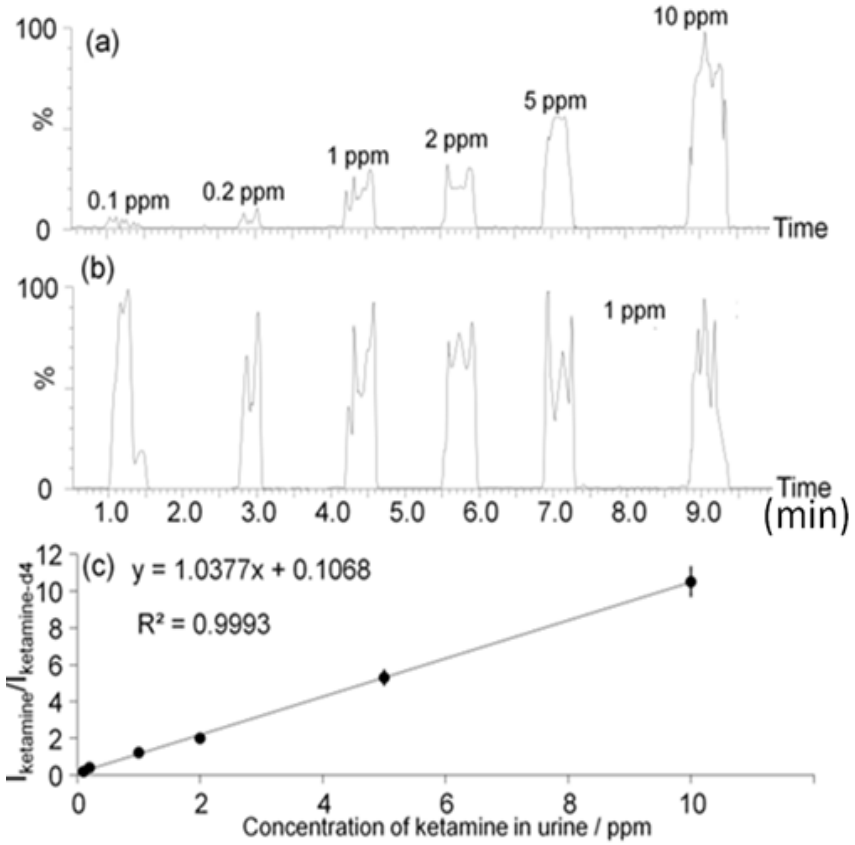
(a)
100
%
0
0.1 ppm
0.2 ppm
1 ppm
2 ppm
5 ppm
10 ppm
Time
(b)
100
%
0
1 ppm
Time
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
(min)
(c) y = 1.0377x + 0.1068
R² = 0.9993
I ketamine/I ketamine-d4
0
2
4
6
8
10
12
Concentration of ketamine in urine / ppm

Figure 5

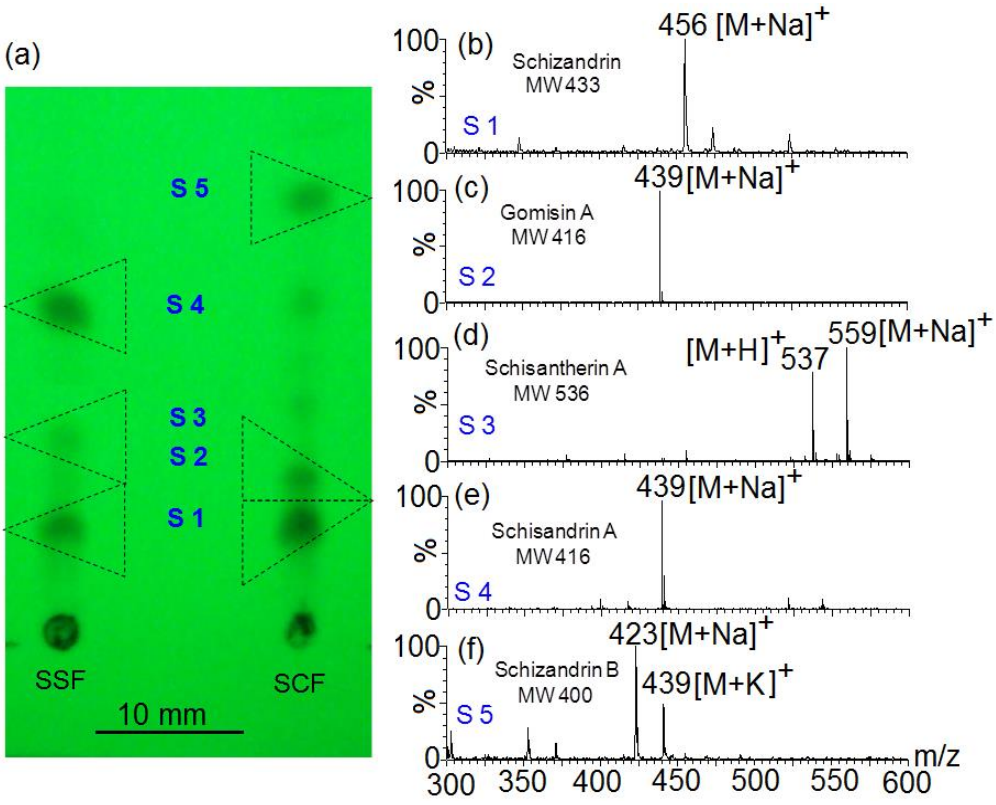
(a)
S 5
S 4
S 3
S 2
S 1
SSF
SCF
10 mm
(b)
Schizandrin
MW 433
S 1
456 [M+Na]+
(c)
Gomisin A
MW 416
S 2
439[M+Na]+
(d)
Schisantherin A
MW 536
S 3
[M+H]+
537
559[M+Na]+
(e)
Schisandrin A
MW 416
S 4
439[M+Na]+
(f)
Schizandrin B
MW 400
S 5
423[M+Na]+
439[M+K]+
100
%
0
300 350 400 450 500 550 600
m/z

Figure 6

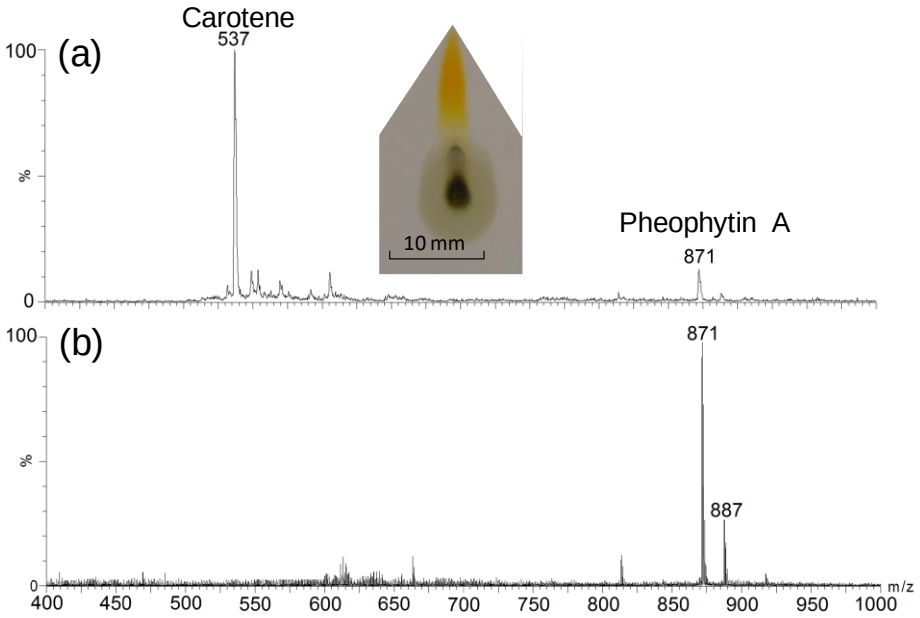
Carotene
537
(a)
100
%
0
10 mm
Pheophytin A
871
(b)
100
871
%
887
0
400
450
500
550
600
650
700
750
800
850
900
950
1000
m/z

Figure 7

Figure captions

Figure 1. Schematic diagram of the TLC-ESI-MS setup.

Figure 2. Mass spectra of 1 μM ginsenoside Rc (GRc) (MW=1079) in different concentrations of NaCl obtained by **(a-e)** TLC-ESI-MS with 10 μL of methanol as the elution solvent and **(f-i)** nanoESI-MS. **(j)** A nanoESI-MS spectrum obtained for 2 μL of solution containing 1 μM GRc and 2 M NaCl and diluted with 10 μL methanol.

Figure 3. Mass spectra of 0.1 mM myoglobin in ACN:water (50:50) containing 0.5% formic acid and 1 mM NaCl obtained by **(a)** TLC-ESI-MS and **(b)** nano-ESI-MS.

Figure 4. (a) & (b) Mass spectra of 50 μM gramicidin D in methanol containing 0.1% formic acid and octly β-D-glucopyranside (5% w/w) obtained by **(a)** TLC-ESI-MS with methanol containing 0.1% formic acid as the elution solvent and **(b)** nano-ESI-MS. **(C) & (d)** Mass spectra of lysozyme in ACN:water (50:50) containing 0.5% formic acid and 5 mM SDS obtained using **(c)** TLC-ESI-MS and **(d)** nano-ESI-MS. Peaks originated from SDS are denoted with "*".

Figure 5 (a) & (b) Selected reaction monitoring (SRM) chromatograms of (a) ketamine (*m/z* 238>125) and (b) ketamine-d4 (*m/z* 242>129) in raw urine samples obtained by TLC-ESI-MS. **(c)** A calibration curve obtained for quantitation of ketamine in raw urine with TLC-ESI-MS.

Figure 6. (a) TLC profiles of extracts of Fruit of *Schisandra sphenanthera* (FSS) and Fruit of *Schisandra chinensis* (FSC) under a 254 nm UV lamp. The spot areas denoted with triangles with dotted lines were cut out for TLC-ESI-MS analysis. The mass spectra obtained for each spot are shown in (b) – (f).

Figure 7. Online TLC-ESI-MS analysis of a spinach extract. Mass spectra obtained for pigments **(a)** carotene and **(b)** pheophytin A.