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Letter to the Editor

## Sensitive determination of picrotoxin by liquid chromatography-quadrupole time-of-flight mass spectrometry

*Dear Editor*

Picrotoxin is an alkaloid found in the berries of *Anamirta cocculus*, a plant of the Menispermaceae family in several regions of Southeast Asia [1]. It is known as an antagonist of glycine receptor and GABA receptor in the central nervous system [2,3], and an equimolar mixture of picrotin and picrotoxinin [4]. Picrotoxin is known as a strong natural toxin and induce convulsion [5]. It has thus been applied as experimental models of epilepsy [3], and the models are used to investigate the biochemical basis of epilepsy and also in the screening of new antiepileptic drugs [5]. Recently, some reports suggest that picrotoxin could be utilized as weapons [6,7]. Jablonski and Jackson reported that picrotoxin was stable in milk after pasteurization [8]. Considering the aforementioned points, it is important to detect and identify picrotoxin with high sensitivity from biological samples. However, despite the widespread use of picrotoxin in studying and treating epilepsy, there is only quite a few reported method for separating and quantifying picrotoxin by liquid chromatography (LC)–mass spectrometry (MS) [8,9].

For detecting and identifying drug and poisons, LC–high resolution mass spectrometry (HRMS) techniques have been applied [10]. Some alkaloids were analyzed by LC–HRMS [11–13]. We report a new method to detect and identify picrotoxin using a novel LC-quadrupole time-of-flight mass spectrometer (Q-TOFMS), one of the LC–HRMS, with high sensitivity and specificity.

Picrotoxin was purchased from Abcam (Cambridge, UK); other common chemicals used were of analytical grade. Urine samples, after obtaining informed consent, were collected from a healthy volunteer. Analyses by Q-TOFMS were employed on an AB SCIEX Triple TOF 5600 system (AB SCIEX, Framingham, MA, USA). The LC instrument used in combination with a Q-TOFMS was a Shimadzu Prominence XR LC system (Shimadzu, Kyoto, Japan). The column used for chromatographic separation was Scherzo SM-C18 (2.0 × 150 mm, particle size 3 μm, Imtakt, Kyoto, Japan). The LC conditions were: flow rate, 0.5 ml/min; column temperature, 40 °C; elution mode, gradient with 5 mM ammonium formate in distilled water (mobile phase A) and methanol (mobile phase B) from 90% A/10% B, after being kept for 1 min, to 5% A/95% B during 4 min followed by isocratic elution with the last solvent composition for 2 min. The tandem MS conditions were: interface, electrospray ionization (ESI) mode; polarity, negative; turbo gas temperature, 500 °C; spray voltage, 4000 V. In the MS mode, ions were scanned in the range from *m/z* 200 to 700; the declustering potential (DP) and collision energy (CE) were 50 V and 10 V, respectively. In the MS/MS mode, a condition for product ion monitoring was settled as follows: precursor ions were 309.1 for picrotin and 337.1 for picrotoxinin, and product ions were scanned in the range of *m/z* 50–390 (DP, 50 V; CE, 30 V). Measurements in MS and MS/MS modes were employed alternatively, for 150 ms and 100 ms, respectively; the total cycle time was 410 ms. For assessing the sensitivity of the instrument, authentic picrotoxin was dissolved in methanol. The concentrations of picrotin was in the range of 0.00013–13 ng/μl, and that of picrotoxinin was 0.013–13 ng/μl in the diluted samples. Two microliters of each solution was applied to the LC-Q-TOFMS system. To evaluate the sensitivity of the method, calibration curves were drawn in the MS mode or the MS/MS mode. In the MS mode, the peak area of *m/z* 309.0870–309.1070 for picrotin, or *m/z* 337.0820–337.1020 for picrotoxinin was measured, respectively. In the MS/MS mode, the peak area of *m/z* 109.0240–109.0340 was measured for both picrotin and picrotoxinin. Calibration curves were drawn three times for each condition, and the accuracies and the coefficients of variation (CV) were estimated for authentic picrotin and picrotoxinin. For evaluation of matrix effects of urine, 200 μl of blank urine was mixed with 100 μl of acetonitrile and centrifuged at 5000 rpm for 10 min. The supernatant was mixed with 700 μl of distilled water containing picrotin and picrotoxinin to give final concentrations of 0.13 or 1.3 ng/μl.

In the MS mode, we succeeded in sufficiently separating picrotin and picrotoxinin on the chromatogram. The retention times for picrotin and picrotoxinin were 3.9 and 4.3 min, respectively (Fig. 1a, b). In the MS negative mode, $[M-H]^-$ ions were observed at *m/z* 309.0980 and 291.0874 for picrotin and picrotoxinin, respectively. In addition, $[M+HCOO]^-$ ions were observed at *m/z* 355.1029 and 337.0929 for picrotin and picrotoxinin. Compared with the sensitivities of $[M-H]^-$ ions and $[M+HCOO]^-$ ions, $[M-H]^-$ ion was better for picrotin, and $[M+HCOO]^-$ ion was better for picrotoxinin. The differences between the observed and the predicted exact mass values of target ions and their three isotopic ions ranged within 1.7 ppm for picrotin, and 4.5 ppm for picrotoxinin. In the MS/MS mode for which DP was 50 V and CE was 30 V, the spectrum for picrotin is presented in Fig. 2 and that for picrotoxinin is shown in Fig. 3, respectively. Possible fragment structures were presumed for some fragment ions (some are shown as insets on Figs. 2 and 3), based on the exact mass values.

As stated previously, we drew calibration curves in the MS mode or the MS/MS mode for the estimation of the lower limits of quantification (LLOQ). In the MS mode, the equation of the curve was $y = 4.60 \times 10^5 x + 44.5$ for picrotin and $y = 2.47 \times 10^4 x - 2.05$ for picrotoxinin. The curves were sufficiently linear in the range of 0.00013–13 ng/μl for picrotin and 0.013–13 ng/μl for picrotoxinin; the coefficient of correlation was 0.994 for picrotin or 0.999 for picrotoxinin. The LLOQ values were estimated when the analyte peak was identifiable, discrete, and reproducible, and the back-calculated concentration had a precision that did not exceed 20%

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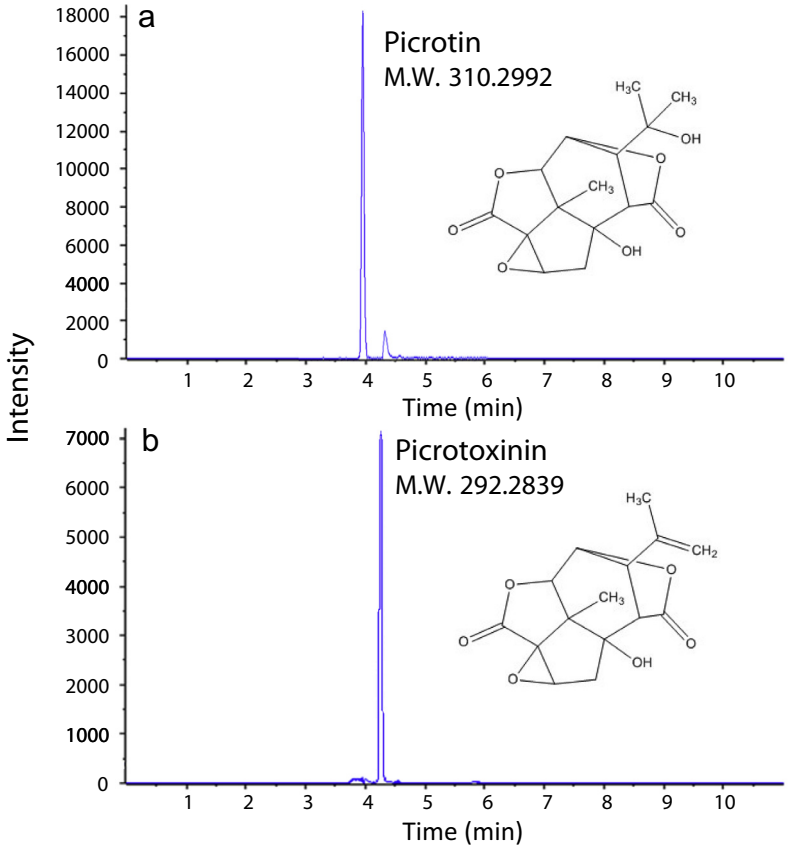


**Fig. 1.** Mass chromatograms of ***a*** picrotin at *m/z* 309.098 and ***b*** picrotoxinin at *m/z* 337.094 in the MS mode; 0.26 ng on-column of each compound was subjected to LC-Q-TOFMS.

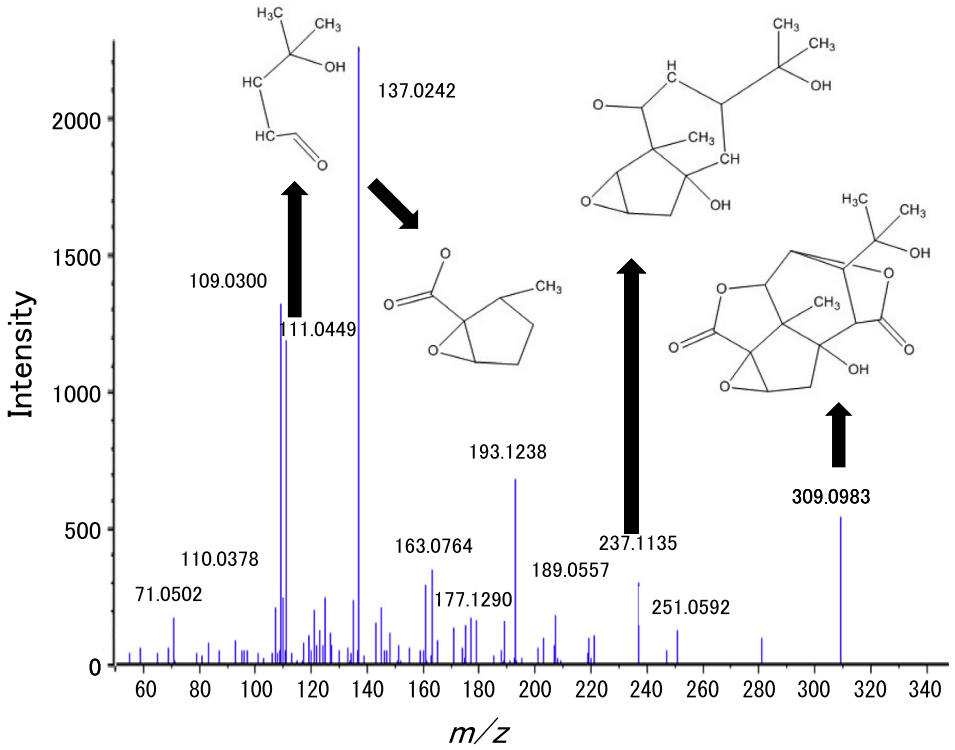


**Fig. 2.** The MS/MS spectrum of picrotin (2.6 ng on-column). Possible structures of fragment ions are shown in the *inset*.