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In Vivo and Real-time Monitoring of Secondary Metabolites of Living Organisms by Mass Spectrometry

Bin Hu^{1,2*}, Lei Wang^{1,2,3*}, Wen-Cai Ye³ & Zhong-Ping Yao^{1,2}

¹State Key Laboratory of Chirosciences, Food Safety and Technology Research Centre and Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong S. A. R., China, ²State Key Laboratory of Chinese Medicine and Molecular Pharmacology (Incubation), Shenzhen Research Institute of The Hong Kong Polytechnic University, Shenzhen 518057, China, ³Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, and Guangdong Province Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, Jinan University, Guangzhou 510632, China.

Secondary metabolites are compounds that are important for the survival and propagation of animals and plants. Our current understanding on the roles and secretion mechanism of secondary metabolites is limited by the existing techniques that typically cannot provide transient and dynamic information about the metabolic processes. In this manuscript, by detecting venoms secreted by living scorpion and toad upon attack and variation of alkaloids in living *Catharanthus roseus* upon stimulation, which represent three different sampling methods for living organisms, we demonstrated that *in vivo* and real-time monitoring of secondary metabolites released from living animals and plants could be readily achieved by using field-induced direct ionization mass spectrometry.

Secondary metabolites play important roles in the survival and propagation of animals and plants. For example, some animals and plants produce chemicals to resist environmental stress, and some secrete toxic chemicals for defense purpose upon attack. Understanding these processes at the molecular level is an integral part of our exploration of nature. Conventional approaches for detection of secondary metabolites typically involve homogenization and extraction followed by analysis of extracts using techniques such as chromatography coupled with mass spectrometry^{1,2}. These approaches are not only time-consuming and labor-intensive, they are also unable to provide transient and dynamic information about the metabolic processes. Techniques for *in vivo* and real-time detection of secondary metabolites from biological systems are thus highly desirable.

Mass spectrometry (MS) is a rapid and sensitive tool for analysis of metabolites from biological samples³⁻⁵. The development of ambient ionization techniques in recent years allows direct analysis of samples with little or no sample preparation⁶⁻⁹. Desorption electrospray ionization (DESI)^{10,11}, direct analysis in real-time (DART)¹², and neutral desorption (ND) coupled with extractive electrospray ionization (EESI)^{13,14} enable direct detection of analytes on surfaces of living organisms at atmospheric pressure. Endogenous compounds of plant tissue could be directly analyzed using techniques such as direct ionization (DI)¹⁵, leaf spray¹⁶ and tissue spray¹⁷. In these techniques, tissue sample is cut into V-shape and connected to a high voltage. With addition of some solvents to the tissue sample if necessary, spray ionization is induced from the tip of the plant tissue and a spectrum is obtained. Leaf spray has been attempted to detect chemical response of cabbage leaf upon punching¹⁶, and DI has also been used for direct analysis of animal tissue¹⁵. These techniques, however, require connection of samples to a high voltage (typically 3–5 kV), which can be a strong stimulus to living plants and animals, and thus may have limited application in *in vivo* stimulation studies of living organisms.

We herein further develop DI-MS for *in vivo* studies of living organisms. In this study, the high voltage required for inducing electrospray ionization is applied to the MS inlet, and the studied animal or plant sample is grounded and placed in front of the MS inlet. To differentiate from the previous DI technique that requires connection of the sample to a high voltage¹⁵, the current technique is termed field-induced DI in this study. We demonstrated that *in vivo* and real-time detection of secondary metabolites released from both living animals and plants upon stimulation could be easily achieved by using field-induced DI-MS.

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Correspondence and requests for materials should be addressed to Z.P.Y. (zhongping.yao@polyu.edu.hk) or W.C.Y. (chywc@yahoo.com.cn)

* These authors contributed equally to this work.

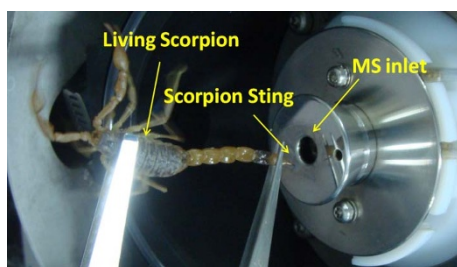


Figure 1 | Photo of *in vivo* analysis of a living scorpion by field-induced DI-MS.

Results

Scorpion uses its sting on the tail as weapon and can release toxic substances from the sting upon attack. Using field-induced DI, spray ionization can be directly induced from such a sharp sting when the sting is placed close and pointing to the MS inlet, and secretion can be conveniently detected (Fig. 1). In this study, a living scorpion was stimulated at its tail with a pair of tweezers, clear liquid secretion was soon released from the sting, and subsequently spray ionization was induced from the sting and a mass spectrum was obtained (Fig. 2). Compound such as lysine (m/z 147), tryptophan (m/z 205) and serotonin (m/z 177) could be identified from the spectrum based on their masses and literature searching^{18–22}. Serotonin is one of the toxins in scorpion to produce pain²¹, and lysine and tryptophan have been reported to play important roles in the biological activity of the scorpion toxin¹⁹. Peaks of high abundances were observed in the range of m/z 400–600. These peaks are singly charged and have a mass interval of 28 Da (e.g., m/z 443.4, 471.4, 499.5 and 527.5, and m/z 487.5 and 515.4) or 16 Da (e.g., m/z 471.4 and 487.5, and m/z 499.5 and 515.4), and may correspond to lipids according to the literatures^{23,24} and the spectral pattern. When the venom was collected and dissolved in common solvents such as methanol, white precipitate was formed. No significant peaks were observed in the spectra obtained by analysis of the supernatant using conventional ESI-MS.

Toad can release venom from its skin for defense purpose upon attack²⁵. In this study, a toad was stimulated at its postauricular gland with a needle (without breaking the skin), and release of white and milky venom was soon observed. A glass capillary containing some methanol was used with one side putting into the secretion and the other side pointing to the MS inlet (Fig. S1). Spray ionization was observed from the capillary tip pointing to the MS inlet and a spectrum was obtained (Fig. 3). Recently, Peng *et al.*²⁶ used a capillary for localized analysis of plants. In their method, a quartz capillary was inserted into a plant for sampling and a stainless needle was inserted into the plant to provide a high voltage to induce spray ionization from the capillary. In this study, with a high voltage applied to the MS inlet, spray ionization was conveniently induced from a glass capillary linked to the sample, and no additional power supply device

connected to the sample was required. This is particularly important and useful for *in vivo* study of living animals since connection to a high voltage in such cases is very inconvenient and can cause adverse effects to the animals and severe perturbation to the stimulation studies.

As shown in Fig. 3, compounds including serotonin ($[M + H]^+$, m/z 177.1), N-methylserotonin ($[M + H]^+$, m/z 191.1), bufotenin ($[M + H]^+$, m/z 205.1), bufotenindine ($[M + H]^+$, m/z 219.1), gamabufotalin ($[M + Na]^+$, m/z 425.2), bufotalidin ($[M + H]^+$, m/z 439.2) and cinobufotalin ($[M + H]^+$, m/z 481.2) could be identified based on masses, MS/MS studies and literature searching (Fig. S3). These compounds were reported as the major compositions of the toad venom, and some of the compounds had been found to have antitumor activity^{27,28}. The secretion became solidified and brown after exposure in the air for about 3 min, indicating some chemical reactions occurred to the secretion. No signals were then observed after solidification of the secretion. It was also noticed that a cluster of ions were observed in the m/z range of 550–700 Da. Venom compositions with masses corresponding to these peaks had not been reported in previous studies, which were mainly based on analysis of dried toad venoms²⁸. Identification of these compounds and studies of their biological functions will be carried out.

Dimeric vinca alkaloids such as vinblastine are powerful anticancer drugs derived from *Catharanthus roseus*. These drugs are produced within the plant by coupling catharanthine with vindoline. Detailed mechanisms for the assembly are still unclear². These dimeric drugs are found at very low levels in the plant and their commercial productions are very expensive²⁹. A recent study by Roepke *et al.*² revealed that catharanthine and vindoline are spatially separated in the leaves of *C. roseus*. It was hypothesized that when the plant is wounded or attacked, catharanthine and vindoline are brought together to allow the formation of the dimers that can prevent cell division. A living *C. roseus* was analyzed using the field-induced DI technique and the spectrum obtained from a leaf of the plant is shown in Fig. 4a. Catharanthine, vindoline and anhydrovinblastine were predominantly observed in the spectrum. When heating at 450°C was induced for 3 sec at ~30 mm away from the spray tip of the leaf, anhydrovinblastine was found to significantly increase, as shown in Fig. 4b for the full spectrum and Fig. S4a for the change in extracted ion chromatogram for anhydrovinblastine. Repeated measurements with three different leaves revealed that abundance of anhydrovinblastine increased from 25.0 ± 4.1% in the spectrum before heating to 93.6 ± 5.2% after heating. Increased abundances of potassium adducts were also observed in the spectrum obtained after the heating, along with the appearance of monogalactosyldiacylglycerol (MGDG) (Fig. 4b and Fig. S4b). Accumulation of potassium has been reported as an osmotic adjustment strategy for plants to maintain water³⁰, and MGDG was previously reported to accumulate when plants were wounded³¹. Our results confirmed the hypothesis proposed by Roepke *et al.*², and suggested that production of anhydrovinblastine in *C. roseus* might be increased by changing the living environment of the plant.

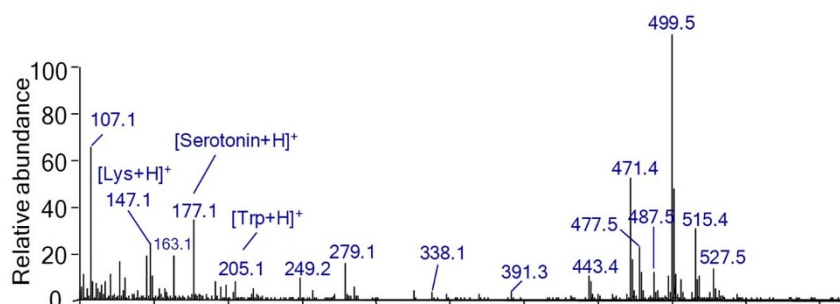


Figure 2 | Mass spectrum obtained by field-induced DI-MS analysis of the secretion released from a living scorpion upon stimulation.

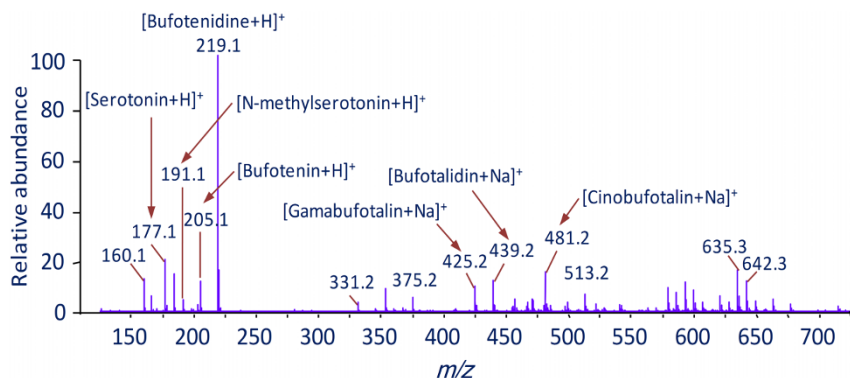


Figure 3 | Mass spectrum obtained by field-induced DI-MS analysis of the secretion released from a living toad upon stimulation.

Discussion

In this study, we presented proof-of-principle for *in vivo* and real-time monitoring of secondary metabolites released from living organisms using field-induced DI-MS. Without connecting the analyzed samples to a high voltage not only avoids perturbation to the stimulation studies and potential hazard to living organisms, but also greatly facilitates sampling from living organisms. Spray ionization could be conveniently induced from a tip of the studied living organism when the tip is moved close and pointing to the MS inlet. Such a tip can be intrinsic to the living organism (e.g., the sting of a scorpion), or become available by introducing a capillary to the sample (e.g., in the case for *in vivo* study of toad), or formed by modifying the sample (e.g., cutting the analyzed leaf to a V-shape). We demonstrated that this technique could be readily used for *in vivo* and real-time monitoring of secondary metabolites released from both living animals and plants upon stimulation. Such *in vivo* and real-time monitoring is simple, rapid, and able to detect compounds that could not be observed by analysis using conventional approaches. Further development of field-induced DI-MS, including allowing observation of more compounds of interest in a single spectrum, coupling it to a high resolution tandem mass spectrometer to

improve identification of compounds, and applying it for studying other interesting biosystems and clinical studies, are on-going.

Methods

Materials. Living scorpion (*Buthus martensii* Karsch; origin: Guanzhou, China), toad (*Bufo bufo gargarizans* Cantor; origin: Guanzhou, China), and *Catharanthus roseus* were supplied by College of Pharmacy, Jinan University (Guangzhou, China). Glass capillaries (ID: 1.0 mm, length: 10 mm) were purchased from Huaxi Medical University Instrument Factory (Chengdu, China). Methanol was purchased from Tedia (Fairfield, OH, USA). The experiments related to animals were approved by College of Pharmacy, Jinan University.

Field-induced DI-MS Measurements. Mass spectra were acquired on an Agilent 6210 TOF-MS instrument (Agilent Technologies, Santa Clara, CA, USA) in positive ion mode. The analyzed biological samples were directly placed in front of the MS inlet with a tip pointing to the inlet. Note that when handling living animals such as scorpion and toad, gloves, mask and goggles should be worn for protection purpose. For field-induced DI-MS analysis of scorpion, a living scorpion was held by tweezers and its sting was directly used as the spraying tip (Fig. 1); for field-induced DI-MS analysis of toad, a living toad was held by hand and a glass capillary, which is commonly used for thin layer chromatography (TLC) spotting, was employed as the spraying tip as described in Results and Discussion and shown in Fig. S1; for field-induced DI-MS analysis of the potted *C. roseus*, the analyzed leaf was held by hand and the spraying tip was formed by cutting the leaf into a V-shape (Fig. S2). The distance between the tip and MS inlet was typically ~ 8 mm. The capillary voltage

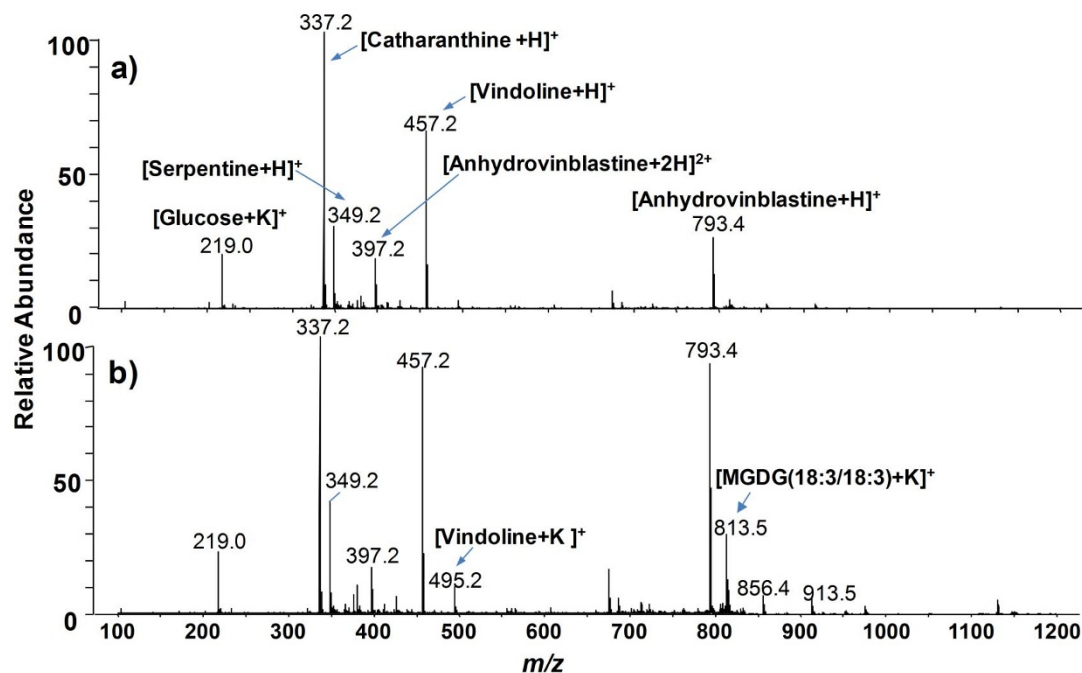


Figure 4 | Field-induced DI spectra obtained for a leaf of a living *C. roseus* (a) before and (b) after heating. Methanol (2 μ L) was used as the added solvent.



(Vcap), that is the voltage applied to the MS inlet, was set at -5 kV for positive ion mode. Drying gas was used with a temperature of 100°C and a flow rate of 4.0 L/min. ESI-MS and MS/MS experiments were performed on a QToF II mass spectrometer (Micromass, UK) for confirmation of peak assignments. Some of the mass spectral results and the experimental details are shown in Fig. S3 and Fig. S5.

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Author contributions

B.H., L.W., W.C.Y. and Z.P.Y. designed the research. B.H. and L.W. performed the experiments and analysed the data. Z.P.Y. wrote the manuscript based on discussions with and contributions from all of the authors.

Additional information

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