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Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 430–436
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Alkaloid profiling in crude and processed *Strychnos nux-vomica* seeds by matrix-assisted laser desorption/ionization-time of flight mass spectrometry

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Received 1 May 2007; received in revised form 20 June 2007; accepted 29 June 2007
Available online 10 July 2007

Abstract

Direct analysis of alkaloids in the tissues of crude and processed *Strychnos nux-vomica* seeds by MALDI-TOFMS was described. The alkaloid profiles of the herb drugs were obtained without the need of complicated sample preparation to avoid potential damage or change of the active components. Seed tissues that were optimally sliced to a thickness of 10–20 μm from the crude and processed *Strychnos nux-vomica* seeds as well as various parts of tissue such as endosperm and epidermis were analyzed on MALDI target plate after the matrix was directly applied onto the tissue surface. The obtained alkaloid profiles provided valuable information for the differentiation of crude and processed *Strychnos nux-vomica* seeds and for the explanation of the significantly different toxicity. Experimental results indicated that the direct MALDI-TOFMS analysis allowed rapid screening of the alkaloid components in *Strychnos nux-vomica* seeds.

Keywords: Direct analysis of seed tissue; Alkaloid profiles; MALDI-TOFMS; *Strychnos nux-vomica*; Semen Strychni

1. Introduction

Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOFMS) has emerged as a great potential technique in the field of biology and medicine for profiling and imaging peptides and proteins directly from thin tissue sections in order to obtain specific information on the local molecular composition, relative abundance, and spatial distribution [1–6]. Direct detection of small molecular weight pharmaceutical compounds in biological tissues [7,8] and plant tissues [9–12] have been reported. Several reviews on the direct analysis of biological tissues by using MALDI-TOFMS have been published [13–15]. The MALDI ion formation process [16–20], MALDI sample preparation procedure [21–24], UV laser focus profiles [25] and matrix selection [26,27] have been studied to improve the technique. MALDI-TOFMS has also been used for the direct analysis of solid sample as a surface analysis technique [28].

Traditional Chinese medicines are gaining increasing popularity worldwide for disease treatment in recent years. Analysis of components in the herbs is essential for the discovery and development of new drugs of natural origin, as well as for quality assurance and toxicological investigations. While most of medicinal herbs have demonstrated none or mild side effects, some herbs may cause toxic side effects if not being properly processed with heating, steaming or soaking. The traditional processing procedures have been proved to be important to reduce the toxicity before the herbs can be used in the prescription of traditional medicines. The roots of *Aconitum carmichaeli* Debx. and the seeds of *Strychnos nux-vomica* L. (Semen Strychni), for example, have to be processed in order to reduce or eliminate the toxic effects of some alkaloids existing in the herbs [29]. It is possible that the toxic components are chemically changed after the traditional process treatment such as heating, soaking or parching, while the therapeutic activities of the herbs remain. Although these crude and processed herbs have been differenti-

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doi:10.1016/j.jpba.2007.06.031

ated and separately used for thousands of years, their remedial mechanism is not well elucidated. Moreover, different herbal processing methods may greatly affect the quality or safety of the medicinal herbs. Conventional analytical methods involving heat extraction using water or solvent and isolation by column chromatography may not ideally suit for the investigation on the difference between the crude and processed herbs because the toxic components may have been changed after the sample extraction. Therefore, it is important to directly analyse the herbal components to support studies on effectiveness and toxicity. The direct analytical method is particularly applicable for toxicological study of original medicinal herbs.

Semen Strychni is frequently used as an important ingredient in traditional Chinese medicines to treat nervous diseases, vomiting, arthritic and traumatic pains [29]. Alkaloids are the main bioactive components in Semen Strychni. A total of 16 alkaloids have been separated and identified from the crude Semen Strychni (Table 1) [30]. Alkaloids in Semen Strychni are known to possess high pharmacological activities [31,32]. However, high dose of the strychnine or brucine may be highly poisonous, and sometimes can cause violent muscular convulsions [33]. Therefore, it is important for the safety appraisal of Semen Strychni. Actually, Semen Strychni needs to be properly processed in order to reduce its toxicity before it is used in the medicinal prescription. The common process procedures include the heating treatment by oil or sand at 240–250 °C for 3–5 min. Studies demonstrated that the contents of the major alkaloids such as strychnine and brucine declined significantly with increased amounts of isostrychnine, isobrucine, strychnine *N*-oxide, and brucine *N*-oxide after the thermal treatments [34].

Several methods have been documented for the determination of strychnine and brucine, including thin-layer chromatography (TLC) [35], liquid chromatography–mass spectrometry (LC–MS) [36], Fourier transform-electrospray mass spectrometry (FT-ESIMS) [37], and capillary electrophoresis–mass spectrometry (CE–MS) [38–41]. The change of the alkaloids has also been measured by conventional methods including TLC [34] and HPLC–ESIMS [42]. The toxicity difference of the crude and processed Semen Strychni was discussed [43,44]. However, the analysis involved the complicated sample preparation including extraction and clean up.

In this paper we employed the direct analysis method for the determination of alkaloid profiles in the seed tissues of crude and processed Semen Strychni and the chemical distribution of various parts of tissues by using MALDI-TOFMS. The obtained data demonstrated that the direct MALDI-TOFMS analysis allowed rapid and reliable characterization of alkaloid components in seed tissues, which is important for the safety appraisal of Semen Strychni.

2. Experimental

2.1. Chemicals and materials

The matrices α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma (St. Louis, MO). DHB was saturated in acetonitrile/water (1:1, v/v) containing 0.1% (v/v) TFA. Saturated CHCA solutions were prepared in different solvent mixtures of 30:70 (v/v), 50:50 (v/v) and 70:30 (v/v) of organic solvent: water with or without 0.1% TFA. HPLC-grade acetonitrile and methanol were purchased from Tedia (OH, USA). Peptide calibration standard II was obtained from Bruker Daltonics (German). The alkaloids strychnine and brucine were purchased from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China); strychnine and brucine *N*-oxide was synthesized according to literature procedures [56]. Crude and processed Semen Strychni were purchased from a herbal market in Guangdong, China and identified by the Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine.

2.2. Preparation of seed tissue sample on MALDI target plate

Semen Strychni was sliced using cryotome (Shandon As 620 cryotome, UK). For endosperm, the middle part of the seed was selected. The epidermis part was sliced in the same way and used for analysis of its chemical components. For both samples, five seed tissues were optimally sliced to a thickness of 10–20 μm so that the majority of the cells in the slice were open and the intracellular contents were exposed. The tissue sample size was about 5 mm × 5 mm. Polymer (10% polyvinyl alcohol and 4% polyethylene glycol) was used to conglutinate the tissue with the caution that the blade did not contact the polymer when the tissue was sliced. The tissue section is gently transferred to a MALDI target plate using forceps. The freshly prepared matrix solution was directly deposited onto the tissue surface using a pipette. The sample plate was located in a vacuum desiccator for several minutes until the fine and uniform crystal distribution was observed under a fluorescence microscope. The tissue samples were then directly analyzed by using MALDI-TOFMS.

2.3. MALDI-TOFMS analysis

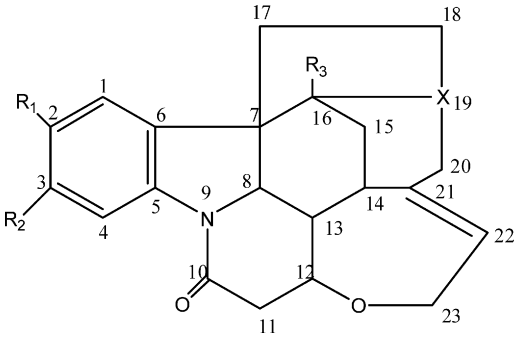
The MALDI-TOFMS experiments were performed on Bruker–Autoflex mass spectrometer (Autoflex, Bruker, Germany). Positive ion reflectron mode was applied with the delayed ion extraction and the delay time was 80 ns. A pulsed nitrogen laser working at 337 nm (pulse energy of 100 μJ) capable of operating at repetition rates of 3–20 Hz was used. The laser beam diameter was about 50 μm and the accelerating voltage was 19 kV. The alkaloid standards strychnine and brucine were loaded with matrix on the same MALDI plate for internal calibration when the seed tissue samples were analyzed. In general, 30 laser shots were averaged for each spectrum and summed over 10 different locations on the target spot.

3. Results and discussion

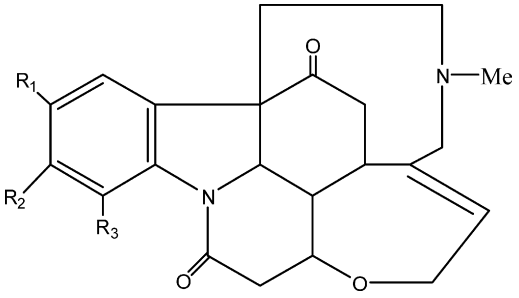
3.1. Direct MALDI-TOFMS analysis of alkaloid profiles on seed tissue

Although the selection of MALDI matrix for the analysis of carbohydrates has been reviewed, there are no general guide-

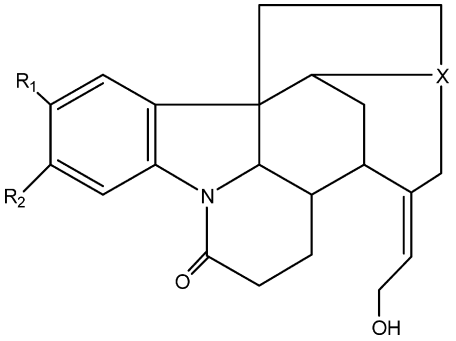
Table 1
Alkaloids identified in Semen Strychni from the MALDI-TOFMS analysis



Name	Formula	Observed $[M+H]^+$	Calculated $[M+H]^+$	R_1	R_2	R_3	X
Strychnine	$C_{21}H_{22}O_2N_2$	335.173	335.175	H	H	H	N
Brucine	$C_{23}H_{26}O_4N_2$	395.195	395.197	OCH_3	OCH_3	H	N
β-Colubrine	$C_{22}H_{24}O_3N_2$	365.180	365.186	OCH_3	H	H	N
Pseudostrychnine	$C_{21}H_{22}O_3N_2$	351.167	351.170	H	H	OH	N
Brucine *N*-oxide	$C_{23}H_{26}O_5N_2$	411.185	411.191	OCH_3	OCH_3	H	N–O
Strychnine *N*-oxide	$C_{21}H_{22}O_3N_2$	351.167	351.170	H	H	H	N–O
Pseudobrucine	$C_{23}H_{26}O_5N_2$	411.185	411.191	OCH_3	OCH_3	OH	N
16-Hydroxy-α-colubring	$C_{22}H_{24}O_4N_2$	381.178	381.181	H	OCH_3	OH	N
2-Hydroxy-3-methoxystrychnine	$C_{22}H_{24}O_4N_2$	381.178	381.181	OH	OCH_3	H	N



Name	Formula	Observed $[M+H]^+$	Calculated $[M+H]^+$	R_1	R_2	R_3
Novacine	$C_{24}H_{28}O_5N_2$	425.220	425.207	OCH_3	OCH_3	H
Icajine	$C_{22}H_{24}O_3N_2$	365.180	365.186	H	H	H
Vomicine	$C_{22}H_{24}O_4N_2$	381.178	381.181	H	H	OH



Name	Formula	Observed $[M+H]^+$	Calculated $[M+H]^+$	R_1	R_2	X
Isostrychnine	$C_{21}H_{22}O_2N_2$	335.173	335.175	H	H	N
Isobrucine	$C_{23}H_{26}O_4N_2$	395.195	395.197	OCH_3	OCH_3	N
Isobrucine-*N*-oxide	$C_{21}H_{22}O_3N_2$	411.185	411.191	OCH_3	OCH_3	N–O
Isostrychnine-*N*-oxide	$C_{23}H_{26}O_5N_2$	351.167	351.170	H	H	N–O

lines for selecting matrix for the analysis of small molecules in plant samples [45,20]. Typical matrixes such as α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) have been used for the small molecules [46–49]. Matrices with high molecular weight matrixes [50,51], the reactive matrix [52], and the inorganic materials [53] have also been applied for the analysis of small molecules in order to reduce the background interference from the matrix.

Common matrices including sinapic acid (SA), 2,4,6-trihydroxyacetophenone (THAP), 3-aminoquinoline, 3-hydroxy picolinic acid (3-HPA), CHCA and DHB were evaluated for the feasibility of the direct analysis of alkaloids in the tissue slice of Semen Strychni. The matrix selection criteria included the number of compounds detected, better signal-to-noise ratio and lowest laser energy needed. The details of the investigations on matrix selection, matrix-to-analyte ratio and laser power for obtaining high-quality MALDI-TOFMS spectra of alkaloids have been reported [12]. It was found that the use of CHCA as matrix produced the best TOFMS signal of the alkaloids. The data indicated that the saturated CHCA solution (~30 mg/ml) in acetonitrile/water (50:50) with 0.1% TFA gave the best results of the alkaloid profiles when the laser intensity was kept as 12%. About 20 μl/cm^2 saturated matrix solution was deposited on the seed tissue for the MALDI-TOFMS analysis.

Fig. 1a showed a MALDI-TOF mass spectrum of the endosperm tissue of crude Semen Strychni. Positive ion mode was used for the detection. The direct tissue analysis provided the TOFMS profile of *Strychnos* alkaloids which are the major bioactive compounds in Semen Strychni. *Strychnos* alkaloid standards strychnine and brucine were used for optimizing the MALDI-TOFMS conditions prior to the experiment on the seed tissue. The obtained data demonstrated that the alkaloids were detected with protonated molecular ions. No sodium or potassium adducts of the alkaloids were observed. Six major peaks at *m/z* 335.173, 351.167, 365.180, 381.178, 395.195 and 425.220 were detected. By comparing the $[M+H]^+$ ions with those of alkaloid standards and compounds reported in reference shown in Table 1 [30], the alkaloids were identified. However, the alkaloid isomers shown could not be differentiated from the TOFMS analysis because no chromatographic separation was involved. The mass assignment on each of the detected alkaloid ions compared to the theoretical values of the reported elemental compositions was achieved with errors of less than 50 ppm. Major components in Semen Strychni were detected by using the direct tissue analysis by MALDI-TOFMS. Strychnine (*m/z* 335) and brucine (*m/z* 395) that are major bioactive compounds in Semen Strychni showed relatively higher intensity compared to other alkaloids. A small peak at *m/z* 351 was observed, which may correspond to either pseudostrychnine, or the *N*-oxide derivative of strychnine (strychnine *N*-oxide or isostrychnine *N*-oxide) according to the reference [30]. The result demonstrated that the direct method which needs no extract preparation might be suitable for detecting trace and unstable compounds in the herbs. However, it should be pointed out that the TOFMS analysis without chromatographic separation could not differentiate isomeric compounds.

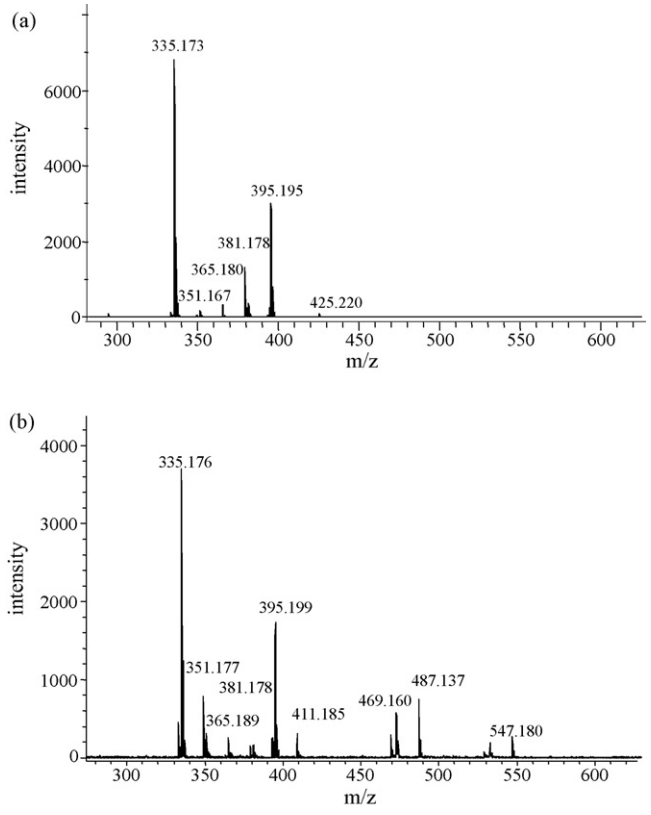


Fig. 1. MALDI-TOFMS spectrum from the direct analysis of (a) endosperm tissue of crude Semen Strychni and (b) epidermis tissue of crude Semen Strychni. Ions at *m/z* 335, 351, 365, 381, 395, 411, 425 were listed in Table 1.

3.2. Detection of alkaloid profiles in tissues from different parts of Semen Strychni

Tissues of different parts such as endosperm and epidermis of crude Semen Strychni were analyzed and differentiated by direct MALDI-TOFMS. When the laser was shot at a certain location on the tissue slice, the detected molecular ions represented the components at the location. Because the structures of most *Strychnos* alkaloids are similar, it is reasonable to assume that they have similar ionization efficiencies and MS responses. Thus, the relative abundances of $[M+H]^+$ ions reflected the relative concentration for each alkaloid, which had been demonstrated by the MALDI-TOFMS analysis of the *Strychnos* alkaloid standards (data not shown). Fig. 1b shows the TOFMS spectrum of the Semen Strychni epidermis. The obtained data showed that the epidermis also contained *Strychnos* alkaloids represented by the ions at *m/z* 335, *m/z* 365, *m/z* 381 and *m/z* 395 (Table 1). The alkaloid profile of epidermis tissue in Fig. 1b, however, was different from that of endosperm shown in Fig. 1a. Several additional alkaloids with higher molecular weights were also observed. Moreover, the spectrum from epidermis tissue showed relatively higher peak intensity of *m/z* 351 compared to that of endosperm in Fig. 1a, indicating that epidermis tissue had higher relative contents of pseudostrychnine or the *N*-oxide derivative of strychnine at *m/z* 351 (strychnine *N*-oxide or isostrychnine *N*-oxide). Analysis of Fig. 1b, the epidermis tissue also contained compounds of pseudobrucine or

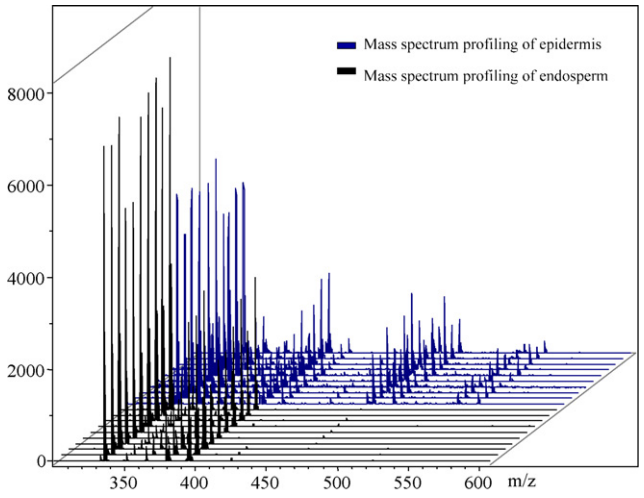


Fig. 2. Stacked view of MALDI-TOFMS spectrum profiles of endosperm and epidermis of crude Semen Strychni. Ten samples were detected for endosperm and epidermis, respectively, and 30 laser shots were averaged for each sample spectrum.

N-oxide derivative of brucine at *m/z* 411 (brucine *N*-oxide or isobrucine *N*-oxide). This result demonstrated that the strychnine and brucine *N*-oxide may already exist in the crude Semen Strychni. The additional alkaloid compounds detected in the epidermis tissue had the molecular ions from *m/z* 450 to *m/z* 550, which were not detected in the spectrum of endosperm. The difference in the alkaloid profiles between the Semen Strychni's endosperm and epidermis could be more clearly demonstrated in Fig. 2. The results from the direct analyses of different parts of Semen Strychni showed the feasibility of using the MALDI-TOFMS method for differentiating the component profiles on various small tissue spots, even from the same herb product. The obtained analytical results might provide useful information for the proper application of Semen Strychni in herbal medicines. The investigation of different chemical profiles on various parts of the seed obviously could not be done with the traditional analytical methods which extracted and analyzed the combined seed samples.

3.3. Differentiation of alkaloid profiles in crude and processed Semen Strychni

According to the regulations authorized by the State Food and Drug Administration of China, Semen Strychni should only be used in the prescription of traditional Chinese medicines after it has been processed with sand heating or oil frying [29]. Pharmaceutical studies have demonstrated that the process may significantly reduce toxic side effects of Semen Strychni [38,39]. It was reported that the traditional detoxification method for Semen Strychni seeds not only removed the fine hairs that could cause throat irritation [54] but also changed the intrinsic alkaloids such as brucine and strychnine into their *N*-oxidative derivatives with less toxicity. Yin et al. [55] and Cai et al. [31] reported that the alkaloids in crude and processed Semen Strychni had the different pharmacological and toxic effects. For example, brucine in crude Semen Strychni was claimed to be a morphine-like analgesic substance, whereas its derivative or brucine *N*-oxide existing in sand-processed Semen Strychni was more a NSAIDs-like compound. The process procedure of crude Semen Strychni has also been shown to have de-toxic effects by oxidizing brucine and strychnine to the *N*-oxide products.

Because the traditional sample extraction procedure prior to the instrument analysis also involved in heating procedure, the alkaloids extracted from the crude Semen Strychni might have undergone the chemical changes after the sample extraction. Direct MALDI-TOFMS analysis of the plant tissue slice, however, could overcome the problems caused by the traditional herb sample extraction. Relative abundances of the $[M+H]^+$ ions of the toxic and less-toxic alkaloids could be determined from the direct analysis of tissue samples. Five processed Semen Strychni samples from different sources were analyzed. The results showed that similar TOFMS spectrum was obtained for the samples from the same processed method (sand heating or oil frying) (data not shown). Fig. 3 shows the TOFMS spectra from the direct tissue analysis of the endosperm from the processed Semen Strychni with sand heating (Fig. 3a) and oil frying (Fig. 3b). Compared to the spectrum of crude Semen Strychni endosperm tissue (Fig. 1a), different profiles of the *Strychnos* alkaloids were observed in crude and processed Semen Strychni. Although strychnine at *m/z* 335 was also detected as the base peak and brucine at *m/z* 395 was showed as the major component in each tissue of the processed Semen Strychni, the peaks of strychnine *N*-oxide or isostrychnine *N*-oxide at *m/z* 351 and brucine *N*-oxide or isobrucine *N*-oxide at *m/z* 411 were clearly

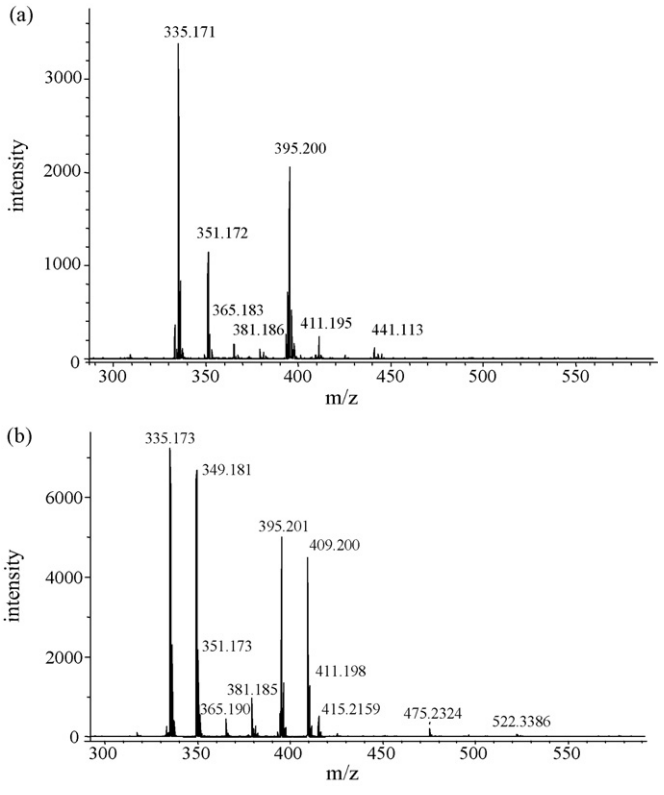


Fig. 3. MALDI-TOFMS spectra from the direct tissue analysis of Semen Strychni (a) processed by oil and (b) processed by sand.

much higher in the spectra of both kind of processed seeds (Fig. 3a and b), indicating that *N*-oxide derivatives were produced after the sand or oil process of Semen Strychni. The results of significantly increased amounts of *N*-oxide derivatives after the herbal process were consistent with the data reported in the reference [36].

The TOFMS analytical results shown in Fig. 3 also indicated that different chemical changes were obtained from the different heating processes using sand or oil. Fig. 3a and b shows different patterns of the alkaloid derivatives produced from the two processes. Strychnine *N*-oxide or isostrychnine *N*-oxide at *m*/*z* 351 and brucine *N*-oxide or isobrucine *N*-oxide at *m*/*z* 411 were detected in both spectra from the sand and oil processed Semen Strychni. In the spectrum of Semen Strychni processed by oil (Fig. 3a), strychnine *N*-oxide at *m*/*z* 351 and brucine *N*-oxide at *m*/*z* 411 were the major peaks, while Semen Strychni processed by sand (Fig. 3b) showed additional major peaks at *m*/*z* 349 and *m*/*z* 409 as the derivatives of the toxic alkaloids. Because the two major ions at *m*/*z* 349 and *m*/*z* 409 were not detected in the tissue sample of crude Semen Strychni, further investigation on the corresponding structures would be of great interest. Nevertheless, the direct analysis by MALDI-TOFMS provided rapid screening and comparison on the toxic alkaloids and their less-toxic *N*-oxide derivatives in the seed tissues of crude and processed Semen Strychni.

4. Conclusions

MALDI-TOFMS is a rapid and straightforward method to generate alkaloid profiles in the seed tissues of Semen Strychni. The method was proved valuable for the direct analysis of crude and processed herbs to avoid tedious extraction and purification steps. The tissue analysis by MALDI-TOFMS prevented the damage of the alkaloid components during the traditional sample extraction procedures. The method can be applied for differentiating the alkaloid profiles in various parts of Semen Strychni such as epidermis and endosperm tissues. The direct analytical method is particularly applicable for toxicity comparison between the crude and processed Semen Strychni from the detection of toxic alkaloids and less-toxic *N*-oxide derivatives. However, separation techniques are necessary to distinguish the isomer compounds and to obtain quantitative data.

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