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NATURAL RESOURCE LETTER

Quality evaluation of Pinellia tuber by LC-TOF/MS targeted to ephedrine

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Abstract
Pinellia tuber (*PTE*, 半夏, 大半夏, 掌葉半夏, 天南星, 水半夏, 半夏瀉心湯, 半夏厚朴湯, 六君子湯) is derived from the tuber of *Pinellia ternata* Breitenbach (Araceae), which is a crude drug used in traditional Japanese Kampo medicine for the purpose of antiemesis and expectoration. Since the separation of ephedrine from *PTE* in 1978, it has been listed as a *PTE* component in textbooks and internet information. Therefore, there are harmful effects on appropriate use in clinical practice because *PTE* is dealt with as a crude drug for doping target, and traditional Japanese Kampo medicine containing *PTE* must be carefully administered to the elderly. However, since the 1978 published report, there has not been any report on the isolation of ephedrine from *PTE* and the interpretation of biosynthesis remains questionable. In the present study, we analyzed the *PTE* samples in market distribution products by LC-TOF/MS. From the analysis of the result of ephedrine's *m/z* 148.113 $[M+H\text{-}H_2O]^+$, *PTE* was not detected ($n=55$, detection limit: 0.5 ppb). Additionally, the tuber of *P. tripartite* (*PTR*, 大半夏), the tuber of *P. pedatisecta* (*PPE*, 掌葉半夏), Arisaema Tuber (*ART*, 天南星), and the tuber of *Typhonium flagelliforme* (*TFI*, 水半夏) that have a similar description to *PTE* were also not detected. Moreover, the genetic analysis of experimental samples showed that *PTE* is derived from *P. ternata*. Furthermore, our attempt to isolate ephedrine from *PTE* based on the past literature was unsuccessful. These results suggest that *PTE* in market distribution products may not contain ephedrine as a component.

Keywords Pinellia tuber · *Pinellia ternata* · Ephedrine · Anti-doping · LC-TOF/MS

Introduction

Pinellia tuber (*PTE*, 半夏) is derived from tuber of the *Pinellia ternata* Breitenbach, and it belongs to Araceae family according to the 17th edition of Japanese Pharmacopeia [1]. It has anti-emetic and expectoration effects [2] and is used as a component for several traditional Japanese Kampo medicine, including hangeshashinto (半夏瀉心湯), hangekobokuto (半夏厚朴湯), and rikkunshito (六君子湯). Phytochemical investigations of *PTE* resulted in the isolation of phenols, phenylpropanoids, flavonoids, polysaccharides, nucleic acids, fatty acids, glycerides, cerebrosides, galactolipids, and sterols [3–5]. Homogentisic acid is the main constituent in *PTE* and is the cause of the acrid taste of *PTE* [6]. The raphides contained in *PTE* is also related to the acrid taste, and a recent study has been reported that it can be removed using ginger extract and lipophilic solvents [7]. In 1978, it was reported that ephedrine was reported from *PTE* [8] and it has been listed as a constituent of *PTE* in textbooks and internet information ever since. However, it has been more than 40 years since the first ephedrine report in *PTE*, but there have been only a few reports [9–11]. Reports of ephedrine in *PTE* can potentially lead to confusion in the educational field, in the practical training of pharmacists, and in clinical practice.

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Ephedrine is an alkaloid isolated from the Ephedra Herb [12] and is used as an agent in bronchodilators, stimulants, and drugs manufactured for the common cold. The World Anti-Doping Agency has published a list of prohibited substances and has classified ephedrine an S6 STIMULANTS banned in sporting competition [13]. Thus, the crude drugs, including ephedrine, are deal with as a doping target material [14]. Additionally, ephedrine is known to cause serious adverse effects, such as palpitation, hypertension, and insomnia. Therefore, ephedrine should be used with caution in patients with heart disease, hypertension, and in the elderly.

Other plant species with tubers similar description to *PTE*, such as the tuber of *P. tripartite* (*PTR*, 大半夏), the tuber of *P. pedatisecta* (*PPE*, 掌葉半夏), Arisaema Tuber (*ART,* 天南星), and the tuber of *Typhonium flagelliforme* (*TFI*, 水半夏) can be mistaken for or mixed into *PTE*.

In this present study, *PTE*, samples obtained from Japanese and Chinese markets, specimen, cultivated, and representative traditional Japanese Kampo medicine were analyzed using ephedrine *m/z* 148.113 $[M+H\text{-}H_2O]^+$ as an indicator by LC-TOF/MS to help construct the evidence necessary for the appropriate use of traditional Japanese Kampo medicine in clinical practice.

Materials and methods

Materials

The crude drug samples analyzed in this work are shown in Tables 1, 2, 3. These samples were purchased from both Japanese and Chinese markets and provided to us by companies and associations (see Acknowledgements). Voucher specimens have been deposited in the school of Pharmacy, Nihon University (Lot No. Anti-doping project 001-071). The crude drug used to prepare Japanese Kampo medicines were purchased from Uchida Wakanyaku Ltd. (Tokyo, Japan). The ephedrine standard (Dainippon Pharma Co., Ltd., Japan) was transferred from National Institute of Health Sciences. The catecholamines and amino acid standards were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) analysis

Each sample (approximately 5–10 g) were boiled in 10 times weight of water for 50 min and filtered, then lyophilized to yield powdered extract. To prepare Japanese Kampo medicines, use the mixture of crude drugs listed in Table 4 were boiled in water (600 mL) for 50 min, and filtered. The decoction was lyophilized to yield powdered extract. The extract and ephedrine standard were dissolved in MeOH to a final concentration of 100 ppm (Some *PTE* samples were prepared at 1000 ppm) and filtered through a 0.45 µm GL Chromato disk 13 A (GL Sciences, Tokyo, Japan) before LC-TOF/MS analysis. The ephedrine standard was further diluted as needed. LC-TOF/MS analysis was performed on a Acquity UPLC coupled to a Xevo G2-S QTOF (Waters, Milford, MA, USA) equipped with an Electrospray ionization (ESI) source. A J-Pak UPX Supero C18 (2.1 mm i.d. × 100 mm, 1.9 µm) (JASCO, Tokyo, Japan) was used for the chromatography at a flow rate of 0.25 mL/min and a column temperature of 40 °C. The injection volume was 2 µL. The mobile phase was composed of A (0.1% formic acid aq.) and B (0.1% formic acid/CH_3CN) with a gradient elution: 0–0.5 min, 0% B; 0.5–4 min 95% B. The qTOF mass spectrometer was operated in ESI positive resolution mode with a capillary voltage of 1.5 kV, and the cone voltage was set to 40 V. The desolvation and cone gas flow were set to 800 and 50 L/h, respectively. The source temperature was set to 120 °C and the desolvation temperature was set to 450 °C. The full-scan mass spectra were collected in continuum mode from *m/z* 100–1000, and SIM mode was collected *m/z* 148.113 $[M+H\text{-}H_2O]^+$, and the data acquisition rate was set to 0.2 s. All MS chromatograms were shown in the supplementary material (Figs 1S-22S).

Confirmed of ephedrine from *PTE* by a method based on past reports

The isolation of ephedrine from *PTE* was performed based on a previous study by Oshio et al. [8], and *PTE* (No. 35, 500 g) was extracted in MeOH at room temperature for 24 h. After evaporation, the MeOH extract was suspended in 0.5 M HCl and partitioned with *n*-BuOH. The aqueous solution was applied to a column of Amberlite IRA 410 (OH^- form, Sigma-Aldrich, St. Louis, MO, USA) to remove Cl^- ions and other acidic substances. After washing the column with H_2O, the non-absorbing eluate was applied to a column of Amberlite IR 120 (H^+ form, Sigma-Aldrich). The column was washed with H_2O and eluted with 5% NH_4OH. Twenty grams of NaCl and five grams of KOH were added to the eluate, and the mixture was stirred. This solution was extracted two times with an equal volume of Et_2O. Thereafter, the obtained Et_2O fraction was analyzed by LC-TOF/MS with ephedrine's *m/z* 148.113 $[M+H\text{-}H_2O]^+$.

Isolation of total DNA and amplification of DNA barcodes

Total DNA was extracted from 2 to 4 g of crude drug material using DNeasy® Plant Mini Kits (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. DNA concentrations and purities were determined by spectrophotometry (Biowave DNA, Funakoshi Co., Tokyo, Japan).

Table 1 Details of the *PTE* samples used in this study and results of LC-TOF/MS analysis

Sample no.	Experimental sample	Place of production	Collection year	LC/MS (100 ppm)
PTE				
1	Specimen	China	1980	N. D.
2	Specimen	China	1980	N. D.
3	Specimen	China	1980	N. D.
4	Specimen	China	1980	N. D.
5	Specimen	Sichuan, China	1993	N. D.
6	Japanese market	Shanxi, China	2011.03	N. D.
7	Japanese market	Shanxi, China	2011.03	N. D.
8	Japanese market	Guizhou, China	2011.04	N. D.
9	Japanese market	Gansu, China	2011.06	N. D.
10	Japanese market	Gansu, China	2012.02	N. D.
11	Japanese market	Gansu, China	2012.04	N. D.
12	Japanese market	Gansu, China	2012.05	N. D.
13	Japanese market	Gansu, China	2012.06	N. D.
14	Japanese market	Gansu, China	2013.04	N. D.
15	Japanese market	Gansu, China	2013.04	N. D.
16	Japanese market	Gansu, China	2013.04	N. D.
17	Japanese market	Gansu, China	2013.12	N. D.
18	Japanese market	Gansu, China	2014.01	N. D.
19	Japanese market	Jilin, China	2014.02	N. D.
20	Japanese market	Gansu, China	2014.05	N. D.
21	Japanese market	Gansu, China	2014.05	N. D.
22	Japanese market	Gansu, China	2014.05	N. D.
23	Japanese market	Sichuan, China	2014.08	N. D.
24	Japanese market	Gansu, China	2014.12	N. D.
25	Japanese market	Gansu, China	2014.12	N. D.
26	Japanese market	Gansu, China	2015.11	N. D.
27	Japanese market	Gansu, China	2016.04	N. D.
28	Japanese market	China	2016.04	N. D.
29	Japanese market	China	2017.04	N. D.
30	Japanese market	Gansu/Sichuan, China	2017.05	N. D.
31	Japanese market	Gansu, China	2017.05	N. D.
32	Japanese market	Chongqing, China	2017.05	N. D.
33	Japanese market	Gansu, China	2017.05	N. D.
34	Japanese market	Gansu, China	2017.05	N. D.
35	Japanese market	Shanxi, China	2017.05	N. D.
36	Japanese market	China	2017.05	N. D.
37	Japanese market	Gansu, China	2017.05	N. D.
38	Japanese market	Guizhou, China	2017.05	N. D.
39	Japanese market	Guizhou, China	2017.05	N. D.
40	Japanese market	Gansu, China	2017.05	N. D.
41	Japanese market	Gansu, China	2017.05	N. D.
42	Japanese market	Gansu, China	2017.05	N. D.
43	Japanese market	Gansu, China	2017.05	N. D.
44	Japanese market	Gansu, China	2017.05	N. D.
45	Japanese market	Gansu, China	2017.05	N. D.
46	Japanese market	Gansu, China	2017.05	N. D.
47	Japanese market	Gansu, China	2017.05	N. D.
48	Japanese market	Unknown	2017.06	N. D.

Table 1 (continued)

Sample no.	Experimental sample	Place of production	Collection year	LC/MS (100 ppm)
49	Japanese market	Unknown	2017.06	N. D.
50	Chinese market	Sichuan, China	2017.07	N. D.
51	Chinese market	Gansu, China	2017.07	N. D.
52	Chinese market	Gansu, China	2017.07	N. D.
53	Chinese market	Sichuan, China	2017.07	N. D.
54	Chinese market	Gansu, China	2017.07	N. D.
55	Chinese market	Sichuan, China	2017.07	N. D.

Table 2 Descriptions of the crude drugs that can be mistaken for *PTE* that were used in this study and the results of LC-TOF/MS analysis

Sample no.	Experimental sample	Place of production	Collection year	LC/MS (100 ppm)
PTR				
1	Cultivated plant	Chiba, Japan	2017.11	N. D.
PPE				
1	Japanese market	Unknown	Unknown	N. D.
ART				
1	Japanese market	Henan, China	1990	N. D.
2	Japanese market	Henan, China	2000.04	N. D.
3	Japanese market	Guangxi, China	2003.05	N. D.
4	Japanese market	Guangxi, China	2010.05	N. D.
5	Japanese market	Guangxi, China	2014.01	N. D.
6	Japanese market	Guangxi, China	2016.06	N. D.
7	Japanese market	Hebei, China	2017.05	N. D.
8	Japanese market	Guangxi, China	2017.11	N. D.
9	Japanese market	Sichuan, China	2018.01	N. D.
TFI				
1	Chinese market	Sichuan, China	2017.07	N. D.

Table 3 Individual parts of *Pinellia ternata* Breitenbach cultivated and the results of LC-TOF/MS analysis

Sample part	Experimental sample	Place of production	Collection year	LC/MS (100 ppm)
Tuber	Cultivated plant	Chiba, Japan	2019. 10	N. D.
Aerial part	Cultivated plant	Chiba, Japan	2019. 10	N. D.
Flower	Cultivated plant	Chiba, Japan	2019. 10	N. D.
Propagule	Cultivated plant	Chiba, Japan	2019. 10	N. D.

Table 4 A list of traditional Japanese Kampo medicine, including *PTE*, that was used in this study and the results of LC-TOF/MS analysis

Kampo	Ingredients	LC/MS (100 ppm)
Hangeshashinto (半夏瀉心湯)	Pinellia Tuber (5 g), Scutellaria Root (2.5 g), Processed Ginger (2.5 g), Glycyrrhiza (2.5 g), Jujube (2.5 g), Ginseng (2.5 g), Coptis Rhizome (1 g)	N. D.
Hangekobokuto (半夏厚朴湯)	Pinellia Tuber (6 g), Poria Sclerotium (5 g), Magnolia Bark (3 g), Perilla Herb (2 g), Ginger (1 g)	N. D.
Rikkunshito (六君子湯)	Atractylodes Lancea Rhizome (4 g), Ginseng (4 g), Pinellia Tuber (4 g), Poria Sclerotium (4 g), Jujube (2 g), Citrus Unshiu Peel (2 g), Glycyrrhiza (1 g), Ginger (0.5 g)	N. D.

Amplification of DNA barcodes and determination of species of the crude drugs were using a modification of the method by Lee et al. [15]. Briefly, PCR was performed using 30–100 ng of total DNA as the template in 25 ml of a reaction mixture containing 2.5 mL 10 × PCR buffer for KOD-Plus- ver.2 (Toyobo, Osaka, Japan), 0.2 mM of each dNTP, 1.0 mM of $MgSO_4$, 0.5 units KOD-Plus-polymerase (Toyobo), and 0.4 mM of each primer. Primers were designed based on the data reported by Lee et al. [15]. Amplification was carried out under the following conditions: pre-heating at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 45 s with a final elongation at 68 °C for 5 min. PCR products were separated using 1.5% agarose gels with a 100 bp ladder marker (Nacalai Tesque, Inc. Kyoto, Japan) and visualized with ethidium bromide (EtBr) staining under ultraviolet light.

HPLC analysis

The *PTE* (No. 2, 4, 10, 13, 18, 31, 32, 33, 41, 45) extract was dissolved in 50% MeOH to a final concentration of 5 mg/mL and filtered through a 0.45 µm GL Chromato disk 13 A (GL Sciences) before HPLC. HPLC analysis were performed on a X-LC system (pump: 3185PU, degasser: 3080DG, mixer: 3180MX, column oven: 3067CO, autosampler: 3159AS, detector: 3110MD; JASCO). A COSMOSIL PBr (4.6 mm i. d. × 250 mm, 5 µm) (Nacalai Tesque) was used for the chromatography at a flow rate of 1.0 mL/min and a column temperature of 40 °C. The injection volume was 5 µL. The mobile phase and monitoring wavelength for adrenaline, noradrenaline, L-DOPA, and dopamine were 20 mmol/L phosphate buffer (pH 2.5), at 280 nm. The mobile phase and monitoring wavelength for L-tyrosine and L-phenylalanine were 0.1% TFA acetonitrile/H_2O (1:4), 220 nm. All HPLC chromatograms were shown in the supplementary material (Fig. 23S and 24S). The L-tyrosine content was determined by the absolute calibration method (Fig. 25S).

Results and discussion

To determine the detection limit of ephedrine, ephedrine standard was analyzed using LC-TOF/MS. The analytical index was determined as *m/z* 148.113 $[M+H\text{-}H_2O]^+$, which is the base peak of the ephedrine MS spectrum. Ephedra Herb was also analyzed to confirm the detection. Figure 1 shows the SIM chromatogram of ephedrine, which is measured in the range of 0.1–1 ppb. From this result, the detection limit of ephedrine was determined to be 0.5 ppb (*S/N* 6.14 ± 1.33, RT 1.88 min).

Next, the *PTE* samples, such as products obtained in the market, specimens, and cultivated plants, were analyzed (Tables 1, 2, 3). Using analysis results from ephedrine's *m/z* 148.113 $[M+H\text{-}H_2O]^+$ as an indicator, ephedrine was not detected in any 55 samples tested (Table 1). Higher concentration samples (1000 ppm) were also analyzed by the same method but were not detected. Additionally, the *PTR*, *PPE*, *ART*, and *TFI* samples were not found ephedrine within the detectable range (Table 2). Genetic analysis was performed on some *PTE* to confirm genetic equivalence, which showed that *PTE* was *P. ternata* (Fig. 2). *PTR* has a most similar in description to *PTE*, it was performed as a comparison target. To examine the possibility that other parts of the plant, besides the tubers, contain ephedrine, the aerial part, flower, and propagule were also analyzed, but ephedrine was not detected (Table 3). Additionally, to examine the possibility of changes in the extracted constituent of *PTE* due to the interaction between other crude drugs and ingredients, we analyzed typical traditional Japanese Kampo medicines that include *PTE*, such as hangeshashinto (半夏瀉心湯), hangekobokuto (半夏厚朴湯) and rikkunshito (六君子湯). However, ephedrine's *m/z* 148.113 $[M+H\text{-}H_2O]^+$ was not detected (Table 4).

Next, ephedrine was extracted with the method of on the report [8]. In this referenced paper, ephedrine (9.5 mg) was isolated from MeOH extract (62.3 g) from 3 kg of *PTE*. Furthermore, fifty milligrams of ephedrine were also obtained from the H_2O extract of the residue. However, the obtained Et_2O fraction by fractionating with past literature was analyzed using LC-TOF/MS using ephedrine's *m/z* 148.113 $[M+H\text{-}H_2O]^+$ as an indicator, and ephedrine was not detected. Therefore, we think that the compound isolated in the previously published report may be contaminated ephedrine, a similar compound, or a precursor of ephedrine.

In a recent study, Fang et al. reported that ephedrine was isolated from *P. ternata* using molecular imprinted polymer coated ionic liquid-based silica [11]. However, the obtained product has not been confirmed by instrumental analysis, and similar compounds, such as catecholamines (adrenaline, noradrenaline, L-DOPA, and dopamine) and its precursors (L-tyrosine and L-phenylalanine), may have been mistakenly detected as ephedrine. Therefore, we examined the catecholamines and their precursor products in *PTE* using HPLC analysis. These results showed that L-tyrosine was contained in *PTE* (aver. 0.662 µg/mg, $n = 10$, RT: 3.39 min), and they suggest that Fang et al. may have detected L-tyrosine and not ephedrine.

Conclusion

In the present study, we employed a highly sensitive method of analysis of *PTE* and similar crude drugs with LC-TOF/MS using ephedrine's *m/z* 148.113 $[M+H\text{-}H_2O]^+$ as an indicator. However, in all the samples examined, we could not to

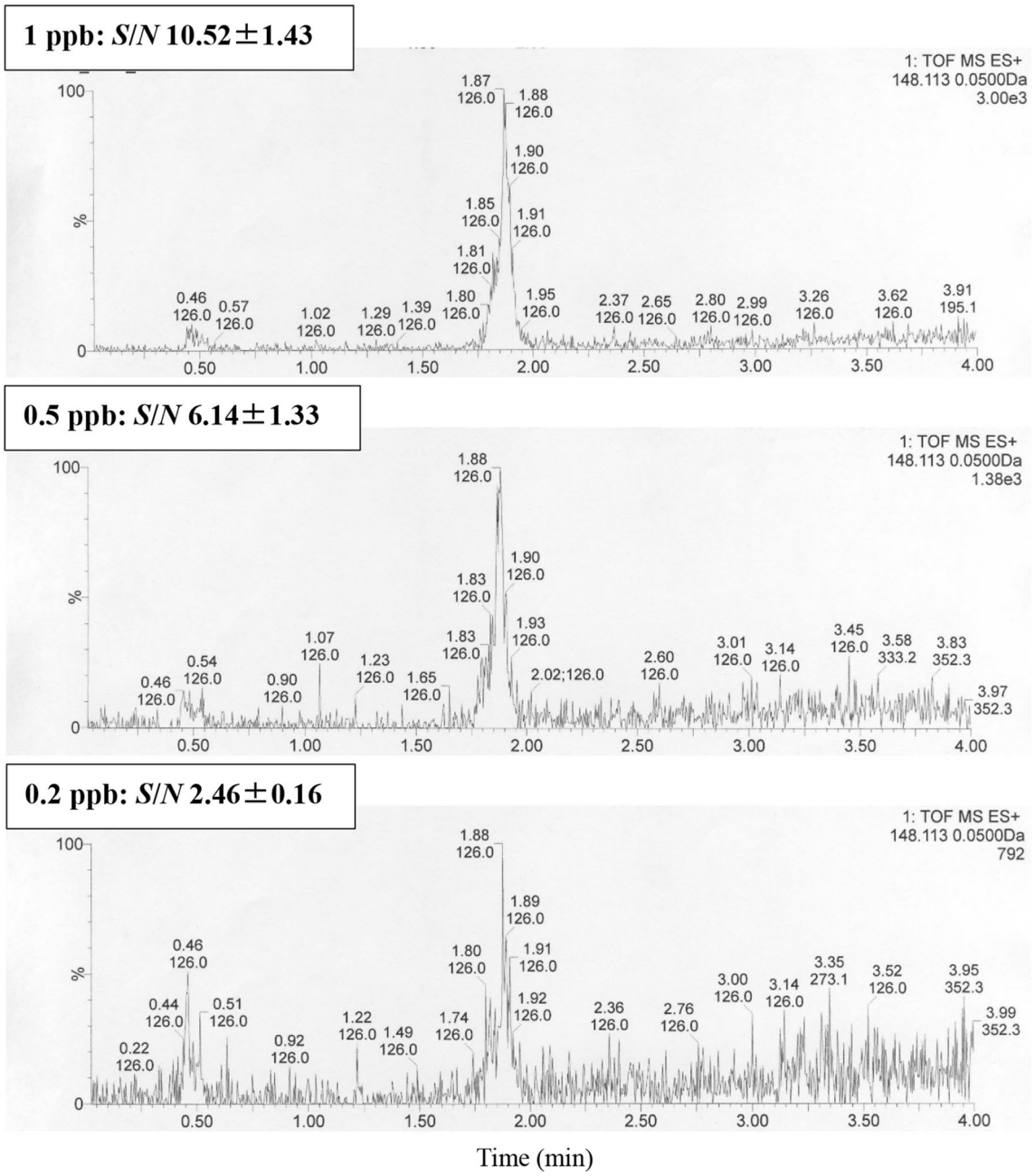

Fig. 1 Detection limit of ephedrine by LC-TOF/MS. *S/N* are expressed as the mean ± S.D. of three independent experiments

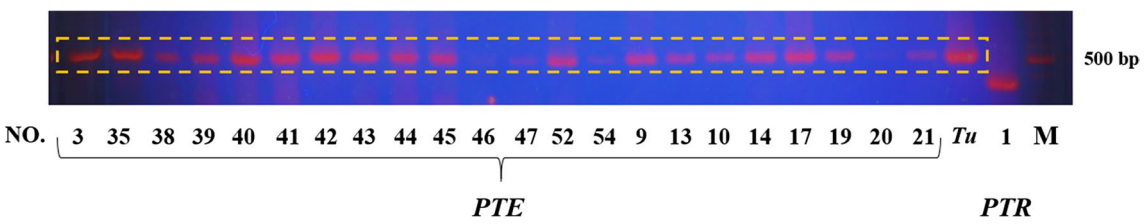

Fig. 2 PCR amplification of ITS1 region. *PTE*, Pinellia tuber; *Tu*, Tuber of *P. ternata* cultivated; *PTR*, *P. tripartite*; M represents the molecular weight of DNA ladder

detect ephedrine within its detection limits (0.5 ppb). These results suggest that *PTE* in distributed market products may not contain ephedrine.

Finally, our results indicate that ephedrine is not a constituent of *PTE*, and hopefully this information can be transferred to the educational materials regarding pharmacognosy and traditional Japanese Kampo medicine, further assisting their proper use in the clinical practice.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11418-021-01485-2.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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