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Quality variation of *maidong* (*Ophiopogon japonicus* and *Liriope spicata*) – A HPTLC-based approach

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ABSTRACT

The tuberous roots of *Ophiopogon japonicus* and *Liriope spicata* are used for the same therapeutic purpose in traditional Chinese medicine and are collectively referred to as *maidong* medicine. Interestingly, it was observed that the price of tuberous roots varies depending on their location on the plant, and fibrous roots are usually discarded post-harvest. Mislabeling might be of concern due to similarities in morphological features between the two species. Moreover, paclobutrazol has been observed to be heavily applied during the production, and therefore might be of health concern. Overall, *maidong* might suffer from quality inconsistencies while its metabolomic complexity is influenced by growing region and cultivation practices, botanical species, and plant parts. To address these challenges, this study employed High-Performance Thin Layer Chromatography (HPTLC) approach, in which sample preparation and derivatization procedure were optimized to enable to capture more detailed and comprehensive metabolomic fingerprints. By integrating with rTLC algorithm and Multivariate Data Analysis (MVDA), an improved quality assessment was achieved. Samples were collected from four production regions and supplemented with commercial products from markets. The optimized HPTLC analysis recognized species- and region-specific metabolomic patterns of *maidong*, uncovering a 4% of mislabelled cases. Moreover, findings highlight the underexplored therapeutic potential of fibrous roots, and comparable therapeutic efficacy between different root types. Additionally, complemented by Liquid Chromatography-Mass Spectrometry (LC-MS) for paclobutrazol residue evaluation, 24.66% of the commercial *maidong* samples surpassed maximum residue limits of paclobutrazol, raising safety concerns. This research represents a significant analytical advancement, offering a robust, cost-effective, and comprehensive method for *maidong* quality control, and paving the way for more strict residue regulation and updates to herbal pharmacopoeias and monographs.

1. Introduction

In Traditional Chinese Medicine (TCM), the tuberous roots of *Ophiopogon japonicus* together with *Liriope spicata* (used as a substitute) are jointly termed as "麦冬" (mài dōng, *maidong*)[1] and are used since ancient times for extending longevity via ameliorating heart-qi stagnation, treating vacuity-taxation, and suppressing vomiting and retching (since ca. 200–250 CE)[2]. Throughout the present study, the term '*maidong*' is used to denote the tuberous roots of *O. japonicus* and *L. spicata*.

Trade practices often lead to (in)advertent mixing of *maidong* due to the roots' morphological similarities. Together with regional production variations across China, such practices contribute to quality inconsistencies. The complexity is further exacerbated by the lack of stringent pre-marketing evaluations and the substantial costs associated with analysis and equipment [3]. Interestingly, it was observed that the price of tuberous roots varies depending on their location on the plant (illustration for root type see Fig. S1) (personal observation, Lei F.Y. (2021)). Tip tuberous roots, formed at the ends of fibrous roots, are preferred over stem tuberous roots that grow near the plant's stem. Despite comprising approximately 40% of the plant's total root weight, fibrous roots are frequently discarded after post-harvest [4], reflecting a divergence in valuation and usage that significantly impacts raw material use and farmer incomes. Therefore, addressing the empirical basis for these practices is essential.

Various metabolomic studies have been conducted to underscore the

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quality variation of *maidong*, by conducting targeted metabolomic analysis with LC-MS, LC-ELSD, RP-HPLC [5–8]. Another chromatographic approach, HPTLC coupled with ^{1}H NMR, was used to determine the metabolomic variance of *O. japonicus* collected in different regions and of different age, based on two saponins (ophiojaponin C & ophiopogonin D)[9]. However, plant crude extracts contain a wide range of substances, which adds to the complexity of spectra generated by NMR, and can greatly increase the difficulty in data interpretation. Moreover, targeted metabolomic analysis might result in biased conclusions, due to the fact that conclusions are drawn based on specific pre-selected molecules. Therefore, it is important to develop a simple, cost-effective analytical method that can provide a comprehensive analysis of the metabolomic fingerprints of *maidong* derived from both, *O. japonicus* and *L. spicata*, and different production regions.

To address these multifaceted challenges, High Performance Thin Layer Chromatography (HPTLC) emerges as a suitable solution recommended by various pharmacopoeias including European Pharmacopoeia and Hongkong Chinese Materia Medica Standards (HKCMMS) [10,11]. High-throughput (15 samples can be screened simultaneously) and cost effectiveness of HPTLC align perfectly with industrial needs of an effective and budget-friendly quality control approach [12,13]. Traditionally, TLC plates have been evaluated by visual inspection, which raised concerns on the inherent subjectivity of visual inspection [14]. The introduction of the rTLC algorithm, developed in the R programming language by Fichou et al. (2016), is groundbreaking in this context [15]. This algorithm processes HPTLC images to eliminate subjective biases and corrects for inter- and intra-plate shifts, representing a significant leap in precision and reproducibility. Furthermore, by integrating pre-processed data with advanced pattern recognition techniques like PCA, this method elevates quality control to a new level.

In the present study, an effective HPTLC analysis protocol was developed to discern metabolomic profile variations in *maidong* from different origins, root types, and mislabeling cases. By leveraging an optimized HPTLC method, integrating it with rTLC and Multivariate Data Analysis (MVDA), we are poised to extract maximal chemometric information from the fingerprints. Additionally, the evaluation of paclobutrazol residues using Liquid Chromatography-Mass Spectrometry (LC-MS) adds a crucial dimension to our understanding of the quality variation in *maidong*.

2. Materials and Methods

2.1. Plant materials

Between February and June 2021, samples were collected in four of the main production regions in China (Fig. S1), including Sichuan and Zhejiang where *Ophiopogon japonicus* is produced, together with Hubei and Shandong where *Liriope spicata* is produced. In total, 155 have been collected from the field of production regions, details about samples can be found in Table S1. Collection was performed in collaboration with and under supervision of Sichuan Agricultural University. Specimens are deposited in the herbarium of the University of Zürich and ETH Zürich (Z + ZT).

After cleaning the plant material, the underground part was separated into three parts, i.e., fibrous roots (FR), tip tuberous roots (TR) and stem tuberous roots (SR) (Fig. S2). Both fibrous and tuberous roots were dried at 60 °C in the oven until it was fully dried, i.e., until the weight stayed unchanged.

Additionally, 73 commercial products were purchased from production regions, Chinese national medicinal markets (An'guo, Bozhou, Yuzhou and Hehuachi), as well as pharmacies which are selling TCM. Among them, 55 samples were classed *as O. japonicus*, and 18 samples as *L. spicata* (Table S2).

All samples were milled using Qiagen Retsch TissueLyser II (20 mm stainless steel ball; 30 Hz frequency; 2 min) and stored in boxes with silica gel for further extraction.

2.2. Chemicals and Reagents

Botanical reference material for *O. japonicus* and *L. spicata* was purchased from the National Institute for Food and Drug Control China.

HPLC grade methanol was obtained from Roth (Germany), dichloromethane and sulfuric acid from Acros (Belgium), and ophiopogonin D from Chemfaces (China).

Paclobutrazol was purchased from EGT Chemie (Switzerland). HPLC grade acetonitrile (Honeywell, Germany), methanol (Sigma-Aldrich, USA), double-distilled water was obtained from Mili-Q. LC/MS grade of formic acid (Fisher chemical, USA).

2.3. HPTLC fingerprint analysis

HPTLC fingerprint analysis was applied on all samples. The method was optimized on the basis of HKCMMS to enhance the efficiency in terms of samples preparation, and maximize the chemometric information retrieved from HPTLC fingerprints [11]. Specifically, three extraction solvents (toluene, methanol, 96% ethanol) were examined to simplify sample preparation process in comparison to HKCMMS; additionally, three derivatization reagents (anisaldehyde, vanillin, sulfuric acid) were compared to enhance detectability and the chromatographic performance, esp. visibility of substances without chromophores or color. Eventually, samples were extracted with 96% ethanol, and derivatization with sulfuric acid. This optimized HPTLC approach improved the comprehensiveness of chemical substances on TLC plates, resulting well-separated zones with good contrasting coloration (see Fig. S3 for HPTLC fingerprints comparison among extraction solvent, and pre-derivatization vs. post-derivatization).

Sample solution was prepared as follows: To 1 g herbal powder, 2.5 mL 96% ethanol was added and sonicated for 10 min at 60 °C. After centrifugation (5000 r/min for 5 min), the supernatant was transferred to 1.5 mL sample vials for later application with the auto sampler.

A CAMAG HPTLC system (Muttenz, Switzerland) was employed, with an automatic TLC sampler 4 (AST 4) for application, an automatic developing chamber 2 (ADC 2) for development, a chromatogram immersion device III for derivatization, a TLC plate heater III, a TLC visualizer 2 for imaging and a software visionCATS 3.1 for data analysis. HPTLC glass plates, 20 cm × 10 cm, silica gel 60 F254 (Merck, Germany) were used.

Application: 5 μL sample solution and reference solutions with ophiopogonin D (500 μg/mL) were applied as 8 mm bands, 11.4 mm apart and 8 mm from the lower edge of the plate. There were 15 tracks per plate with the first application position at 20 mm from the left edge. Universal HPTLC Mix (UHM) was applied on each plate for analytical quality control.

Development: A mixture of dichloromethane, methanol and water (8:2:0.3, v/v) was used as developing solvent. Prior to the development, the plates were exposed for 10 min to a relative humidity of 33% using a saturated $MgCl_2$ solution. The saturation time was 20 min with filter paper, and the developing distance from the lower edge was 70 mm.

Derivatization: Derivatization was performed by immersion into 10% (v/v) sulfuric acid in methanol (speed 4, dwell time 0).

After derivatization, the plate was heated for 3 min at 105 °C. Plate images were recorded before and after derivatization in 366 nm UV light. All instruments and procedures were controlled by visionCATS 3.1.

2.4. Paclobutrazol investigation

2.4.1. Series standard solution preparation

Paclobutrazol was accurately weighed and added with methanol to produce a stock solution with concentration of 0.2654 mg/mL. Then serial standard solutions were prepared by pipetting at the following concentrations: 0.048, 0.104, 0.199, 0.398, 0.796, 1.592 μg/mL. The standard calibration curve was made based on the dilution serial

standard solutions.

2.4.2. Sample extraction

0.5 g of powder samples was added with in 5 mL MeOH. The samples were sonicated for 30 min at room temperature and centrifuged for 10 min (5000 rpm, 4 °C). The supernatants were then transferred into Eppendorf tube and stored at 4 °C fridge overnight for second time centrifugation (14,000 rpm; 4 °C; 15 min), eventually supernatants were collected into 1.5 mL vials and stored at − 20 °C prior to analysis.

2.4.3. High-Performance Liquid Chromatography Coupled with Mass Spectrometry Analysis

Samples were analyzed by HPLC-MS using an Agilent series 1260 Infinity II HPLC system (Waldbronn, Germany) coupled to an Agilent series InfinityLab LC/MSD single quadrupole mass spectrometer with electrospray ionization (ESI). Chromatographic separation was achieved on a Zorbax SB-C18 2.1 × 50 mm column with 1.8 μm pore size (Agilent, Switzerland), maintained at 40 °C. Injections of 2 μL were eluted at a constant flow of 0.6 mL/min with a gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as follows: (i) 40% to 50% B from 0.0–2.0 min; (ii) rapid increase to 100% B and hold for 2 min as wash phase; (iii) followed by 2 min reconditioning at 40% B.

Mass spectrometry data were acquired using selected ion monitoring (SIM) in positive mode to detect paclobutrazol with an expected mass of $[M + H]^+ = 294.1$ *m/z*. The fragmentor voltage was set to 70 V, with a nitrogen drying gas flow of 12.0 l/min at a temperature of 350 °C, and the ESI capillary voltage set to 3 kV.

2.4.4. Quantification of paclobutrazol

A quadratic regression of the calibration curve using the paclobutrazol reference standard was used to determine the concentration of paclobutrazol in each sample. Linear regression analysis showed calibration curve to be linear within the concentration range for paclobutrazol.

2.5. Statistics

HPTLC images obtained from VisionCATS were exported to an open source rTLC (http://shinyapps.ernaehrung.uni-giessen.de/rtlc) for chromatogram extraction and data pre-processing [15]. The parameters used for chromatogram extraction were the same as those used for sample application on the plates, with a pixel width of 128. Data was pre-processed and warped with 'dtw' method to overcome inter- and intraplate band shifts. Eventually, the HPTLC fingerprints were transformed into data matrix of three color's channels (red, green, and blue). Data matrix from gray channel (the mean value of red&green&blue channels) were used as input for multivariate data analysis (MVDA). MVDA was performed using mixOmics package [16], and visualized with ggplot in RStudio [17].

ArcGIS pro (3.1.2) was used for map design.

3. Results & Discussion

3.1. HPTLC fingerprint analysis of the metabolomic profile

3.1.1. Optimized HPTLC analytical protocol to facilitate fingerprints representation and interpretation

Chromatographic and mass spectrometric techniques are pivotal in herbal medicine quality control [18]. Traditional analyses often rely on pre-selected markers, which, while useful, can be biased, potentially overlooking adulteration with similar substances [19]. Our approach overcomes this limitation by integrating HPTLC and rTLC, which allows for an untargeted analysis, providing a comprehensive view of the metabolomic profiles. HPTLC's automation and high-throughput nature (screening 15 samples simultaneously) ensure both cost-effectiveness and reproducibility, with subsequent MVDA facilitating pattern recognition [3,20].

In the present study, we streamlined the time-intensive sample preparation procedure described in the HKCMMS by employing sonication and testing three extraction solvents to ensure the representation of *maidong*'s metabolomic fingerprints. Furthermore, we optimized visibility of the zones by experimenting with three derivatization reagents. The resulting optimal HPTLC procedure involved extraction with 96% methanol, chromatographic development on silica gel 60 F_{254} HPTLC plates using a dichloromethane, methanol, and water (8:2:0.3, v/v) system, followed by derivatization with 10% (v/v) sulfuric acid in methanol (see Fig. S3 for HPTLC fingerprints comparison among extraction solvent, and pre-derivatization vs. post-derivatization).

To mitigate the subjectivity associated with traditional TLC visual inspection and address variations from mobile phase, humidity, temperature, operator handling, and instrument stability, we applied a standardized chromatogram extraction protocol[15]. Dynamic time warping (dtw) was utilized to correct for inter- and intraplate band shifts, as illustrated in Fig. S4, showcasing the densitograms before and after correction. This precise correction facilitates a more scientific and objective analysis, enabling the accurate deciphering of maidong's metabolomic variations.

3.1.2. Maidong from different botanical and geographical origins

Based on visual inspection of HPTLC fingerprints of *maidong* derived from different botanical and geographical origins (Fig. 1), a yellowish zone due to a steroidal saponin- Ophiopogonin D was observed in *maidong* derived from *Ophiopogon japonicus* and *Liriope spicata*, and most intensely expressed in *maidong* derived from *O. japonicus* in Sichuan. Apart from Ophiopogonin D, it was obvious that the metabolomic pattern of *maidong* derived from *O. japonicus* (Fig. 1A: 4&5) and *L. spicata* (Fig. 1A: 2&3) is distinct at inter-species level. A light yellow zone at Rf 0.25 (yellow arrows) and a blue zone at Rf 0.52 (red arrows) were observed on the fingerprints of *O.japonicus* extracts, whereas they were absent in *L. spicata* extracts. At intra-species level, fingerprints of *maidong* derived from *O. japonicus* in Sichuan differed from those in Zhejiang. For instance, an intense yellowish zone at Rf 0.21 was observed in *maidong* produced in Sichuan due to Ophiopogonin D. Moreover, at Rf 0.41, a blue zone (green arrow) presents itself in *maidong* produced in Sichuan, whereas a yellow zone (green arrow) showed up in *maidong* produced in Zhejiang. However, *L. spicata* produced in two different production regions showed slight differences by visual inspection of the fingerprints.

Unsupervised principal component analysis (PCA) showed clear separation among *maidong* derived from different origins (Fig. 1B). Data matrix generated from rTLC was applied. It is demonstrated that PC1 (principal component 1) explains 69.34% of the variance, and PC2 explains 10.66% of the variance. Totally, the first two components can explain 80% of all the variance, indicating this model preserves good predictivity.

The PCA results correspond to the pattern we observed through visual inspection on the chromatograms (Fig. 1A),

1) At inter-species level, metabolomics of *O. japonicus* and *L. spicata* are distinctively different, as two 95% confidence interval (CI) ellipses of each species includes most of the individuals,
2) At intra-species level, metabolomics of *O. japonicus* and *L. spicata* differ between production regions, respectively. This is reflected by the clear separation according to production region.

The distinct differences at an inter-species level were clearly observed and can be credited to the differences in metabolomics, e.g., different composition of steroidal saponins [21]. *L. spicata* as another important source of *maidong*, its metabolomic variation was barely explored so-far in literature. Our expanded HPTLC analysis was applied, for the first time, on both *O. japonicus* and *L. spicata*. Ophiopogonin D, as a recognized chemotaxonomic marker for *O. japonicus* in HKCMMS, was

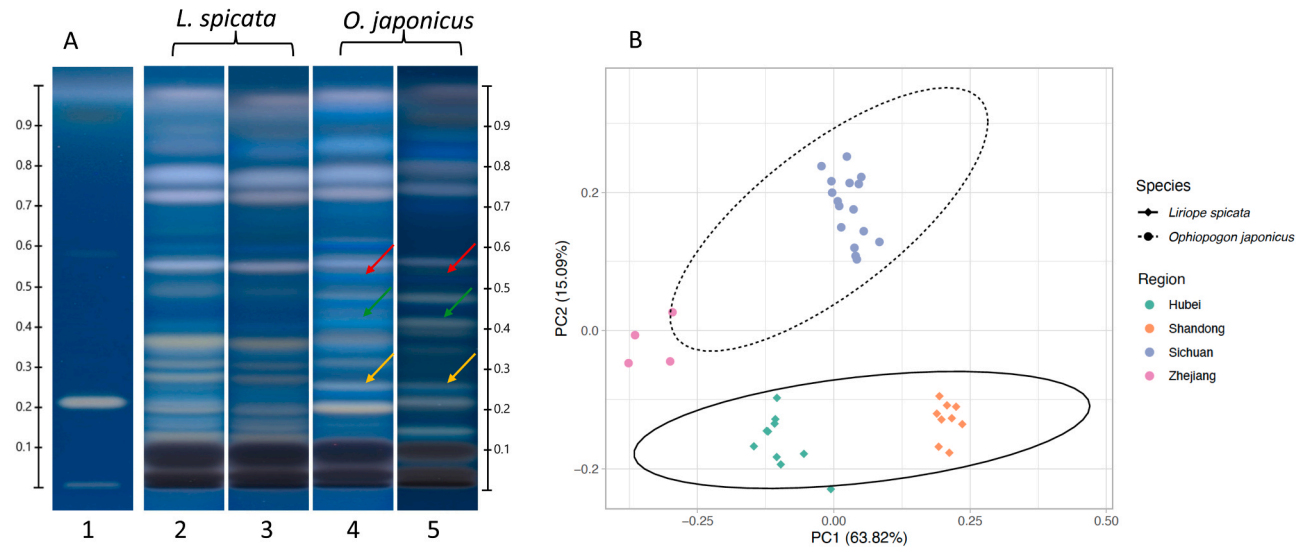

Fig. 1. HPTLC fingerprint of exemplary *maidong* of different botanical and geographical origins (A) (1, reference: Ophiopogonin D; 2, *L. spicata* from Hubei; 3, *L. spicata* from Shandong; 4. *O. japonicus* from Sichuan; 5, *O. japonicus* from Zhejiang), and PCA plot based on the metabolomic profile of all samples analysed (B) (Shapes differ from botanical origins: dot represents *L. spicata* individual, triangle represents *O. japonicus* individual; Colors differ from production regions: Hubei is in green, Shandong is in orange, Sichuan is in violet, Zhejiang is in pink).

detected in both species, indicating that using ophiopogonin D as the sole discriminant is insufficient as interspecific marker. Moreover, fingerprints of *maidong* derived from *L. spicata* in Hubei and Shandong resembled to each other based on visual inspection, but, were successfully distinguished by PCA based on the data matrix obtained using rTLC algorithm. Overall, this highlighted the necessity for our untargeted HPTLC approach, which can provide a comprehensive view of the metabolomic landscape. By processing TLC images with rTLC and applying MVDA, we were able to undertake a more thorough fingerprint analysis, thus ensuring a robust evaluation of *maidong* quality based on a comprehensive spectrum of its phytochemical complexity.

Clear chemotypic divergences are evident between *O. japonicus* from Sichuan and Zhejiang, similar pattern was observed also by Ge et al. (2019) and Jiang et al. (2022)[5,9]. This may be associated not solely to the different geographical location but also to different agricultural practice in the production regions. In Zhejiang, the production of

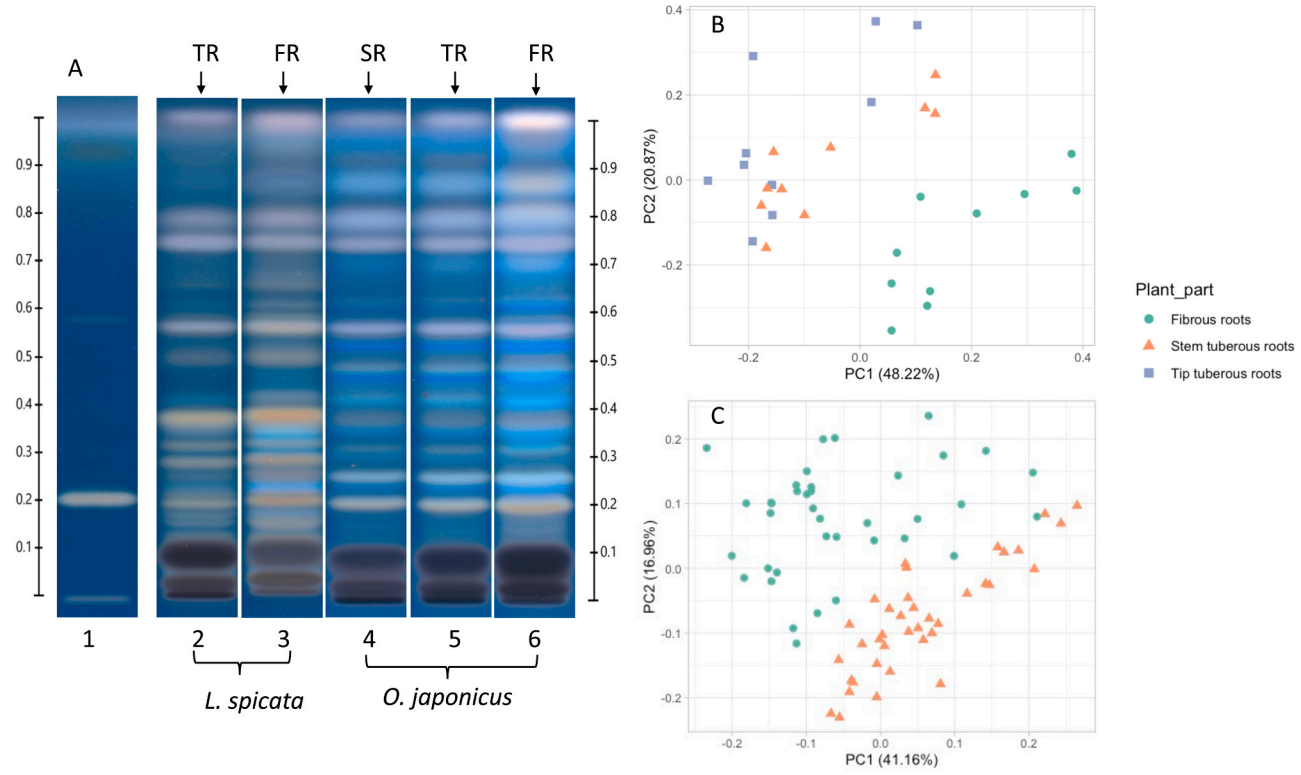

Fig. 2. HPTLC fingerprint of exemplary of different root types of *L. spicata* and *O. japonicus* (A) (1, reference: Ophiopogonin D; 2–3, TR and FR of *L. spicata* from Hubei; 4–6, SR, TR and FR of *O. japonicus*), and PCA plot on the metabolomic profile of different root types from *O. japonicus* (B), PCA plot on the metabolomic profile of different root types from *L. spicata* (C).

O. japonicus requires a three-year period prior to harvest, and no paclobutrazol is applied during the cultivation. In Sichuan, *O. japonicus* is cultivated for one year prior to harvest, with maize being intercropped during the initial stage (personal observation, Lei F.Y. (2021)). It was also noteworthy that Ophiopogonin D content in *maidong* derived from *O. japonicus* in Sichuan is higher than that in Zhejiang, and higher than in *maidong* derived from *L. spicata*, which is in accordance with Ge et al. (2019) and Lyu et al. (2020) [9,22].

3.1.3. Comparing different root types

HPTLC fingerprints of different root types of *Ophiopogon japonicus* and *Liriope spicata* were summarized in Fig. 2A. Overall, chromatograms of fibrous root extracts and tuberous root extracts showed similar patterns from a qualitative perspective. However, quantitatively speaking, more intense zones were observed in fibrous root extracts. This is similar to the findings that same chemical constituents were observed both in fibrous roots and tuberous roots, whereas more abundantly accumulated in fibrous roots, such as total saponins, total flavonoids, and polysaccharides (Chen et al., 2022; He et al., 2012; Qi et al., 2015; Wu et al., 2016; Zhou et al., 2019).

Moreover, chromatograms of stem tuberous roots (SR) extracts (Fig. 2A (4)) mirrored with tip tuberous roots (TR) (Fig. 2A (5)) by visually evaluating the HPTLC plate image at UV 366 nm.

An unsupervised PCA was performed, and the first two components explained 69.09% and 58.12% of the variance in the PCA model of *O. japonicus* and *L. spicata*, respectively, enabling the model to predict the metabolomic pattern. PCA clearly separated tuberous roots from fibrous roots of *O. japonicus* (Fig. 2B) and *L. spicata* (Fig. 2C). However, SR and TR were barely separable, which was also reflected by visual inspection on HPTLC chromatograms. This revealed their similarity in metabolomic profiles, suggesting that SR and TR may have comparable therapeutic efficacy.

3.1.4. Commercial samples

Commercial *maidong* samples were subjected to HPTLC analysis. Unsupervised PCA was not able to discriminate commercial *maidong* samples from their botanical origins (Fig. 3A), revealing the complexity of commercial *maidong*. Therefore, a supervised model sPLS-DA (Sparse PLS-discriminant analysis) was performed to increase the predictivity of the model by maximizing the covariance between variables, and selecting a small subset of the most informative predictor variables (Fig. 3B). Declared botanical species were used as discrete factors. The sPLS-DA model greatly improved the separation between *O. japonicus* and *L. spicata*. Variance explained by the first two components (71.84%) indicated good prediction ability of this model. Two samples declared to be *O. japonicus* were clustered together with *L. spicata* samples, one sample was on the edge of 95% CI of *O. japonicus* but very close to *L. spicata* samples. Eventually, potential mislabeling or substitution was recognized by the protocol - three out of seventy-three (4%) were mislabeled.

Historically, *maidong* has been sourced from multiple botanical origins including *O. japonicus* and *L. spicata* among others [2,23,24]. However, modern pharmacopoeias, including HKCMMS as well as European Pharmacopoeia, solely recognize *O. japonicus* as the accepted botanical source for *maidong* [10,11]. Given the reduced market value of *L. spicata* compared to *O. japonicus*, the potential for intentional mislabeling or adulteration is possibly driven by profit motives.

Moreover, it is noteworthy, that in the process of trading *maidong*, a showcase of intentional substitution raised concern, i.e., substitute *O. japonicus* with *L. spicata*. Middlemen sometimes received custom orders seeking tuberous roots that bear an extremely close morphological resemblance to those of *O. japonicus* (personal observation, Lei F.Y. (2021)). The intentional substitution during the early stage of trade makes the supervision harder, thus pre-marketing assessment is highly recommend to ensure product purity in the marketplace and underscores the intricate dynamics at play in *maidong* trade.

In the present study, an unsupervised model, PCA, was sufficient to discriminate *maidong* from different populations. This indicates that the chemometric traits retrieved from the HPTLC fingerprints well represent the metabolomic variance. This shows that our integrated approach with HPTLC, image processing by rTLC and MVDA worked efficiently. While PCA can efficiently distinguish HPTLC fingerprints of *maidong* derived from different botanical species, it failed to discriminate commercial *maidong* samples of different botanical origins. Only a more robust predictive model, i.e., sPLS-DA, which maximizes the separation between/among predefined groups and selects subsets of the most informative variables, successfully recognized mislabeling cases.

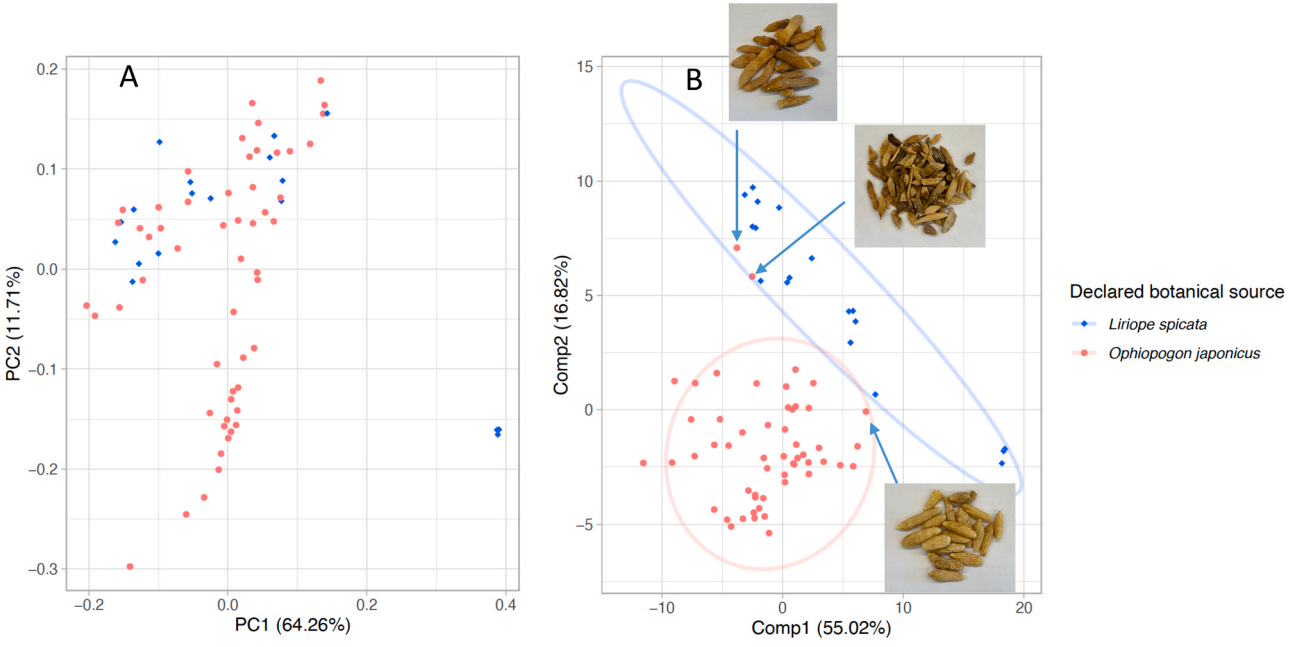

Fig. 3. Multivariate data analysis on the metabolomic profile of commercial *maidong* samples (A. PCA score plot, B. sPLS-DA score plot).

3.2. LC-MS analysis on paclobutrazol residue

Paclobutrazol as a plant growth regulator has been applied on crops to increase the yield. However, excessive application of paclobutrazol can cause health issues. Carcinogenicity, reproductive toxicity, digestive system toxicity, hepatotoxicity, teratogenicity, and neurotoxicity have been reported for paclobutrazol [25–28]. Moreover, studies have shown that it can significantly decrease the content of steroidal saponins (e.g., Ophiopogonin D) and flavonoids (e.g., ophiopogonanone C) which are considered responsible for *maidong's* therapeutic efficacy [29]. Unfortunately, paclobutrazol has been heavily used during *maidong* production, especially in Sichuan.

Currently, the content of paclobutrazol is not regulated and monitored in medicinal plants and their products, but only in food crops, vegetables and fruits. According to EFSA (2022), the paclobutrazol MRL (maximum residue limit) for fruits like apples, pears, or quinces is 0.05 mg/kg [30]. Considering *maidong* is the product of dried tuberous roots, we thus converted this MRL based on the drying rate of *maidong* (70%). Therefore, the MRL for paclobutrazol was set as 0.17 mg/kg as a pragmatic tool to assess the risks of contamination, but without a toxicological assessment per se.

Among all the samples collected in the field, paclobutrazol was only detected in *maidong* produced in Sichuan (botanical source: *O. japonicus*). Therefore, the paclobutrazol residue among different root types of *O. japonicus* produced in Sichuan was investigated. The results of 10 *O. japonicus* individuals were summarized in Fig. 4. Fibrous roots tend to possess the highest concentration of paclobutrazol residue ranging from 0.18 to 4.03 mg/kg, followed by stem tuberous roots, and tip tuberous roots. This resembles the pattern observed for other metabolites, i.e., fibrous roots were generally found to show higher concentrations both of metabolites and paclobutrazol. Noteworthy, paclobutrazol residues in most of the tuberous roots of these 10 samples were below the MRL, whereas the residue in the majority of the fibrous roots exceeded the MRL.

Furthermore, samples purchased from markets were investigated on paclobutrazol residues. This suggests an excessive use of paclobutrazol, especially in products derived from *O. japonicus* (Fig. 5). Overall, 24.66% (18 out of 73) *maidong* samples were below the MRL. All *maidong* samples derived from *L. spicata* were below MRL, even barely detectable, whereas most of the *maidong* derived from *O. japonicus* had detectable levels. Notably, one sample derived from *O. japonicus* contained paclobutrazol concentrations far beyond the MRL, with a peak value of 2.18 mg/kg (Fig. 5).

4. Conclusions

This study presents an advanced untargeted metabolomic approach using High Performance Thin Layer Chromatography (HPTLC) to investigate the complex metabolite profiles of *maidong*, specifically from *O. japonicus* and *L. spicata*, across four production regions. Our refined HPTLC protocol, featuring optimized extraction and derivatization processes, represents a significant analytical enhancement, enabling the capture of more detailed and comprehensive metabolomic fingerprints.

Through the integration of multivariate data analysis (MVDA), our approach not only recognizes clear species- and region-specific metabolomic patterns but also identifies mislabeling. In the batch of 73 commercial samples examined, 3 (4%) were found to be incorrectly labelled, signalling the robustness of our method in detecting adulteration and misidentification in the market. Moreover, the underappreciated metabolomic richness of fibrous roots compared to tuberous roots was revealed, opening new avenues for therapeutic application, although the detected paclobutrazol warrants cautious utilization.

Furthermore, no substantial difference in the metabolomic profiles between stem and tip tuberous roots were found, affirming their equivalent therapeutic uses. This analytical insight challenges traditional perceptions and supports a more inclusive approach to using *maidong* roots.

In conclusion, the HPTLC technique applied in this study offers a simple, cost-effective, robust, and reproducible method for the quality control of *maidong*, outperforming traditional practices with its comprehensive analytical scope. It underscores the need for more strict regulatory measures for residue levels in herbal preparations, advocating for updated standards in pharmacopoeias and herbal monographs. The study's methodological advances set a new benchmark for the precision and reliability of herbal medicine analysis.

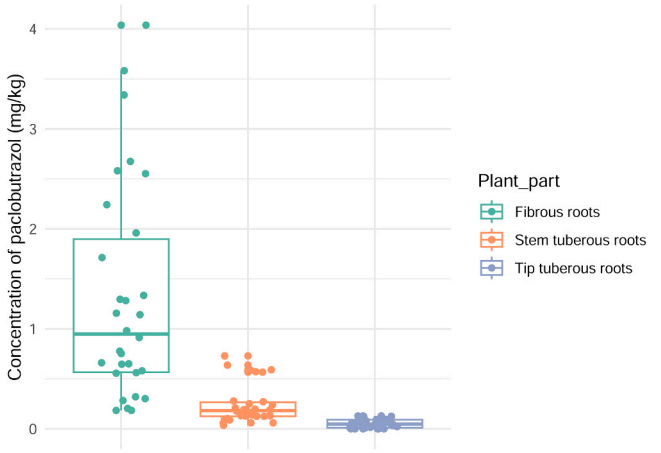

Fig. 4. Paclobutrazol concentration among different root types of *O. japonicus*.

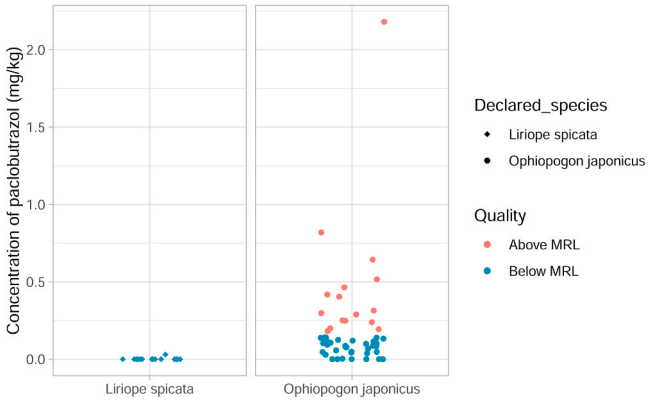

Fig. 5. Paclobutrazol concentration of purchased commercial samples (Green indicated paclobutrazol content was below the MRL (<0.17 mg/kg), red indicated paclobutrazol content was above the MRL (≥0.17 mg/kg).

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CRediT authorship contribution statement

Reich Eike: Methodology. **Weckerle Caroline Sonja:** Conceptualization, Writing – review & editing. **Lei Feiyi:** Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Heinrich Michael:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper. Eike Reich worked for CAMAG, the producer of the HPTLC system, and was actively approached by the other authors for technical support. All other authors declare no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2024.115990.

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