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Review

Mass spectrometry imaging for unearthing and validating quality markers in traditional Chinese medicines

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ABSTRACT

Quality marker (Q-Marker) is an innovative concept and model for quality control of Traditional Chinese medicines (TCMs), which will navigate the new direction of quality development of TCMs. Yet, how to characterize the overall quality attributes of TCMs and their biological effects is still debating. In view of this key scientific issue, this paper proposes a research method based on mass spectrometry imaging (MSI) technology for the discovery and confirmation of TCMs Q-Marker. MSI is powerful in investigating the spatial distribution of molecules in a variety of samples, and visualizing the information obtained from MS. On this basis, combine with the five principles of TCMs Q-Marker validation, i.e., specificity, transmission and traceability, testability, prescription compatibility, and validity, were applied to confirm the finalized Q-Marker. It will lead the new direction of quality development of TCMs.

Contents

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1. Introduction

Traditional Chinese Medicines (TCMs) are a major component of China's traditional medical technology and culture, and has been the main material means for the Chinese people to prevent and treat diseases for thousands of years. It has the distinctive features of "multi-component, multi-target, multi-pathway and multi-action", but the pharmacological components of many TCMs are still uncertain. Scientifically and comprehensively controlling and evaluating the quality of TCMs is a key bottleneck and technical difficulty in the modernization of TCMs development. The quality control (QC) methods of TCMs should strive to achieve the technical specifications of exact content, clear structure, and unambiguous efficacy. At present, the active ingredients of TCMs are difficult to be thoroughly elucidated, and the technical level of TCMs quality control is far from the characteristics of the material system of TCMs, all of which make it difficult for the current TCMs quality control and evaluation model to scientifically and comprehensively control and evaluate the quality of TCMs (Xiao, Jin, Zhao, Xiao, & Wang, 2007).

Nowadays, most commonly used methods for quality evaluation of TCMs are high performance liquid chromatography (HPLC) (Yan & Wu, 2016), gas chromatography (GC) (Hong, Wang, & Zhu, 1991) and their combination with mass spectrometry (MS) (Li et al., 2018; Yue, Zuo, Huang, & Wang, 2021), which proved useful in quanlitative and quantitative determination of index components. Nevertheless, problems such as poor correlation between QC indexes and the effectiveness of TCMs, and poor specificity persist, which hinder the modern QC of TCMs. Moreover, TCMs is a synergistic combination of multiple components with poly-pharmacological effects, and the content of a single or several index components can hardly reflect the panorama of TCMs quality (Pan, Wang, & Yang, 2020).

In 2016, Academician Liu Changxiao's team proposed the concept of TCMs quality marker (Q-Marker), which has the basic characteristics of specificity, effectiveness, measurability, and relevance to the TCMs theory; it is a new concept and model for QC of TCMs plants and products, which inspires a new development direction for the TCMs quality, and is important in promoting the modernization and internationalization of TCMs (Liu et al., 2016; Liu, 2021).

In recent years, with the rapid development of modern instrumental analysis technology and the rise of artificial intelligence (AI) technology, the quality control and evaluation mode of TCMs is gradually shifting from relying on traditional experience, detection of main active ingredients and index components to multiple index components, "one measurement, multiple evaluation", fingerprinting, biomarkers, and others. Many new technical means of quality control and evaluation are emerging, and the approaches of discovering Q-Marker of TCMs are gradually changing from active ingredient detection to the flexible applications of network pharmacology (Li, He, et al., 2021; Fan et al., 2021), chemometrics (Bai et al., 2019; Jiu, Zhang, Liu, Liu, & Wang, 2021), molecular docking (Zhang et al., 2021; Zhu, Peng, Jiang, Mai, & Zhang, 2021) and other means of prediction, as well as fingerprinting (Ni, Lian, Zhao, Li, & Guo, 2021) and other methods for corroboration, to name a few. Such methods mostly focused on strategies, and it is difficult to really reach effective corroboration of Q-Markers of TCMs.

Mass Spectrometry Imaging (MSI) is a novel technique for visual analysis and detection, which has been recognized as an effective tool for detecting and identifying complex molecules in samples (Li, Ji, & Gao, 2023; Miyamoto et al., 2016), and is widely used in numerous fields such as life sciences, biomedicine and environmental toxicology. MSI can play an important role in the composition study of TCMs, and its visualization and high resolution characteristics will contribute to the study of TCMs Q-Marker, just like *Isatidis Radix* (Banlangen in Chinese) (Nie et al., 2022) and so on.

How to discover and confirm Q-Marker of TCMs is a difficult problem that has not been effectively solved and needs to be studied in depth. MSI, as an attractive approach, is highlighted and scrutinized in this paper.

2. Quality control of TCMs and TCMs Q-marker

2.1. Current development of TCMs quality control

In the early development of TCMs, quality control of TCMs often relied on the initial sensory evaluation of morphological characteristics such as appearance shape, color, and odor of medicinal materials; with the advancement of science and technology, the chemical evaluation model of TCMs quality was established to identify and quantify the index components of TCMs. Currently, the QC model of TCM has evolved from evaluation of single component index to the overall QC model of active ingredients, characteristic components, fingerprinting, etc. However, thus far the single index is still commonly used, and poor correlation and difficulty in covering medicinal effects are still problematic, making it difficult to guarantee the quality control of TCMs (National Pharmacopoeia Committee, 2013).

2.2. Proposal of Q-marker of TCMs

The upsurge of Chinese medicine industry calls for the consistent quality of TCMs products and systematic and stable quality standard system, so as to achieve precise QC of Chinese medicine and ensure the efficacy and credibility of Chinese medicine. The QC and evaluation are a key scientific issue in the modernization and development of TCMs, as its intrinsic quality directly affects the clinical efficacy and safe use of TCMs.

In light of the complexity of TCMs theory, compounding, drug preparation, dosage form processing and usage, Liu proposed the concept of TCMs Q-Markers (Liu et al., 2016; Zhang et al., 2018a; Zhang et al., 2018b), which are chemical substances inherent in Chinese herbal medicines (CHMs) and Chinese medicine products (e.g., TCMs tablets, tonics, extracts, preparations, etc.) or formed during processing and preparation. These compounds are closely related to the functional properties of TCMs and can be used as markers to reflect the safety and efficacy of TCMs (Liu et al., 2016).

2.3. Basic requirements and discovery principles of TCMs Q-marker

The TCMs Q-Markers should be: (1) specialized metabolites inherent in TCMs herbs and products, or chemical substances formed during processing and preparation; (2) chemical substances that are solely present in a herb (and/or its TCMs decoction pieces) and not from other herbs; (3) substances with clear chemical structure and biological activity; (4) substances that can be identified qualitatively and quantified; (5) substances that are representative of the "ruler" herbs, e.g., "monarch", "minister", and "assistant" herbs, according to the principle of the preferred "ruler" herbs in a TCMs formula (Zhang, Bai, & Liu, 2019). The validation of Q-Marker should be based on the "five principles" of validity, i.e., specificity, transmission and traceability, measurability, prescription dispensing, and validation of candidate TCMs Q-Markers (Fig. 1) (Zhang et al., 2018a; Zhang et al., 2018b; Zhang, Bai, & Liu, 2019).

2.3.1. Validity

In TCMs, "validity" is the core element of Q-Marker. The understanding of "effectiveness" in the theoretical system of TCMs is based on the "potency" and "efficacy" of drugs, and the change process of the body should also be considered to reach the understanding of "efficacy", "property" and "process in the body". The *in vivo* exposure and pharmacokinetics of TCMs chemical groups are the material basis for the expression of "effectiveness" of TCMs. For example, NHBA, an anti-insomnia candidate isolated from *Gastrodia elata* Bl. (Tianma in Chinese), was analyzed by DESI-MSI in the total frozen section of rats, which showed that NHBA mainly distributed in the intestinal tract of rats, a small amount of NHBA accumulated in the brain, and six endogenous metabolites changed significantly after administration, including gamma- aminobutyric acid, choline, valine, etc. Increased levels of these compounds can better explain the sedative and hypnotic effects of NHBA (He et al., 2015).

MSI was applied to examine the absorption, distribution, metabolism, elimination (ADME) and pharmacokinetics of candidate TCMs Q-Marker in the body, to clarify the role and distribution of candidate Q-Marker, and to explore the distribution tendency and expression of their biological effects, which is an important basis for confirming the Q-Marker of TCMs.

Fig. 1. Five principles for discovery and determination of Q-Marker in TCMs. Through the application of different experimental methods, we closely combined the five principles of TCMs Q-Marker discovery, namely validity, specificity, testability, prescription compatibility, transmission and traceability mining the Q-Marker of TCMs.

2.3.2. Specificity

At present, there are many medicinal materials in the quality standard of TCMs that take the ubiquitous components (e.g., chlorogenic acid, rutin, etc.) as the content measurement indexes for the QC. These indices cannot reflect the characteristics of different TCMs. The chemical substances in TCMs have diverse types and complex structures, which may come from different medicinal materials or different medicinal parts of the same medicinal materials. The same/similar chemical composition is often related to the genetic background of species, source pathway, ecological/geographic conditions and other factors.

For example, in the research process of *Areca* nut, *Areca* nut is the dried seed and the areca nut is the dried peel. The visualization of chemical composition in *Areca* nut was carried out by applying DESI-MSI, and it was found that *Areca* nut contains abundant alkaloids, such as *Areca* line, while the *Areca* line does not contain alkaloids. This study highlights the uniqueness of the only alkaloid component in palm Chinese medicine (Srimany et al., 2016). The "specificity" of chemical components of TCMs is particularly important in the identification of TCMs Q-Marker. MSI can be used to directly characterize the chemical components of TCMs and their tissue distribution, which helps clarifying the specificity of Q-Marker. The biogenic pathway of secondary metabolites can be analyzed according to the MS spectrogram, and the characteristics of TCMs such as kinship, uniqueness of harvest period and biological growth period, and ecological environment can be taken into account altogether. The specificity of chemical substance groups of TCMs must be clarified, so as to provide evidence for confirming TCMs Q-Marker.

2.3.3. Transmission and traceability

After multiple process optimization, such as collection, preliminary processing, further processing, extraction and preparation, and transportation and metabolism in the body, the composition of the really effective TCMs chemical group is quite different from the original chemical group.

Using MSI, the chemical groups of TCMs in each process of processing, process optimization and metabolism *in vivo* can be characterized. The "chemical components" (Nie et al., 2021), the "change components" (Li et al., 2020a; Li et al., 2020b) after processing, the "original components" (Xue et al., 2018) of dosage form optimization, and the "effective components" (Meng et al., 2020) that play the function in the body were analyzed. It is necessary to clarify the transfer and change process of chemical substance group of TCMs, which is an important basis for confirming Q-Marker.

2.3.4. Testability

"Testability" is a necessary prerequisite for the discovery of TCMs Q-Marker. The TCMs components are complex and diverse, and the drug properties, efficacy, usage and dosage vary greatly under different conditions. If components, as the candidates of Q-Marker, cannot be effectively measured, they are easily ignored with the change of conditions. For example, *Salviae Miltiorrhizae Radix et Rhizoma* (Danshen in Chinese) is a commonly used TCMs in promoting blood circulation, removing blood stasis, relieving pain, and reducing inflammation. Tanshinone IIA, a major efficacy compound of Danshen, acts on key targets of blood stasis such as thrombin and plasminogen activator inhibitor-1 to expand arteries and reduce blood viscosity (Ma, Chen, Bian, Chen, & Qiao, 2020). Then damaged vascular endothelial cells can be repaired to promote blood circulation and remove blood stasis. In the inflammatory model, it was found that salvianolic acid components inhibited inflammatory cytokines such as TNF-α and IL-6 (Wu et al., 2020). Undoubtedly the testability of Tanshinone IIA and salvianolic acids must be guaranteed, as these components are potential Q-Markers. MSI can not only determine the content of components, but also obtain the molecular composition *in vivo*, relative abundance and spatial distribution of components. Compared with GC–MS and LC-MS, MSI can better determine the expression of complex and diverse components of TCMs *in vivo*. This is an important prerequisite for confirming Q-Markers.

2.3.5. Prescription compatibility

The TCMs formulas are frequently used in the clinical setting, so the prescription compatibility is also an important problem to be faced in confirming TCMs Q-Marker. The same medicinal material can often play distinct roles in different prescriptions, which not only includes the changes in the combination of prescription and syndrome such as "sovereign, minister, assistant", but also shows that the complex TCMs components can play corresponding roles in different environments. According to the confirmation principle of TCMs Q-Markers, the "sovereign medicinal" in the formula should be taken as the preferred principle, and the representative substances of "minister", "assistant" and "guide" drugs should be taken into consideration, so as to clarify the TCMs Q-Markers for different symptoms.

DESI-MSI was applied to study the QC of Shaoyao Licorice Decoction (Qu et al., 2020) and Banxia Xiexin Decoction (Sun et al., 2020), etc., the content of main drug components in these fromulas was determined, and the difference between single decoction herb and decoction per se was compared. It laid the foundation for confirming Q-Markers of TCMs in the formula.

3. Impact of MSI technology

3.1. MSI

It is a label-free technique that enables the visualization of chemical components in cells and tissues at high spatial and quality resolution. MSI integrates MS and visual imaging analysis, which is able to detect biomolecules based on "molecular mass fingerprinting" and simultaneously analyze tens to hundreds of different compounds, such as lipids and specialized metabolites. In MSI, each mass signal is mapped to the location where it was recorded through specialized software tools, and these data are reconstructed into a three-dimensional molecular image, which can reflect the "volume-effect" and "time-space" changes of the components in the body in a multidimensional and highly specific manner. The "time-space" changes in the organism are essential for compound identification and "on-line" structural characterization (Dreisewerd & Yew, 2017; Miyamoto et al., 2016).

The first step of MSI is to acquire and prepare the sample to be measured in a suitable way, which is followed by a predefined acquisition procedure, and scanning the sample using laser or high-energy ion beam, etc., in order to desorb and ionize the molecules or ions on the sample surface. Then, the mass-to-charge ratio and ion intensity of the ions are obtained at each pixel point on the sample surface by a mass analyzer, followed by searching for the mass spectral peaks of the relevant mass-to-charge ratio ions in the mass spectral data of each pixel point with the help of relevant software, combining the signal intensity of their corresponding ions, and processing the continuous sections of the sample to obtain the three-dimensional spatial distribution of the substance to be measured in the sample (Buchberger, Delaney, Johnson, & Li, 2018).

3.2. MSI category

As a "molecular microscope", MSI can include Desorption Electrospray Ionization MSI (DESI-MSI), Matrix Assisted Laser Desorption Ionization MSI (MALDI-MSI), and Secondary Ion MSI (SIMS) depending on the mode of ionization (Table 1, Fig. 2). DESI-MSI is an open-access MSI technique that is easy to operate, and the sample analysis process is not dependent on the spray matrix. In the detection of substances in the low mass region, it is of high sensitivity and wide coverage (Thoma, 2017). DESI-MSI was applied to study the metabolism and lipid profile of various types of renal tissues while also diagnosing the differences between renal tumor subtypes that could not be distinguished by histopathological analysis, i.e., renal eosinophilic cell tumor and suspicious renal cell carcinoma, which played a great role in the clinical treatment (Zhang, Li, Lin, Yu, & Eberlin, 2020). DESI-MSI and DESI/PI were combined to distinguish the differences in lipids between different groups; when combined with multivariate statistical analysis, cholesterol was visually identified as a biomarker for human melanocytic nevi (Qi et al., 2021).

MALDI-MSI is the most widely used imaging technique in MSI studies, with broad applicability and flexibility for visualizing lipids, peptides, proteins, metabolites, and drugs (Schulz, Becker, Groseclose, Schadt, & Hopf, 2019). MALDI-MSI relies on tissue sections coated with a homogeneous matrix layer that absorbs the energy of the pulsed laser, which is usually emitted in a horizontal grating on the sample, allowing analyte desorption/ionization. The disadvantage of MALDI is that sample preparation is more complex, as a homogeneous matrix coating is required to ionize the sample and the analyte of interest needs to be extracted onto the tissue surface and co-crystallize with the matrix (Kompauer, Heiles, & Spengler, 2016). The integrated morphological image analysis was performed to characterize and classify single cell-specific molecular profiles of individual cells by MALDI-MSI, a

Table 1
Comparison of MSI methods.

MSI methods	Resolution	Environment	Advantage	Disadvantage	Application
DESI-MSI	50–500 μm	Atmosphere	Simple sample pre-treatment	Low spatial resolution, not conducive to non-polar material detection	Observation of biological small molecules such as metabolites, drugs and lipids
MALDI-MSI	About 50 μm	Atmosphere or vacuum	Extensive coverage	Complex matrix background leads to limited spatial resolution	Biological tissue sectioning to observe larger biomolecules in tissue
SIMS	50 nm–5 μm	Vacuum	High spatial resolution	Low ion yield and easy fragmentation of analytes	Imaging surface elements and molecular composition at the cellular scale

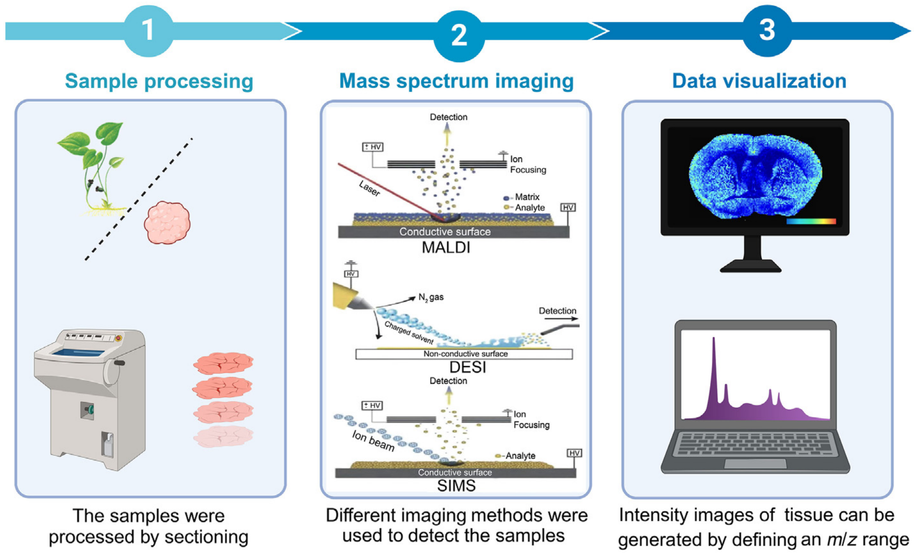

Fig. 2. Processes and applications of MSI. After the samples were processed by different methods, the samples were analyzed by mass spectrometry and visual data through MSI (MALDI, DESI, SIMS).

method that provides a new means to study spatial biological processes such as cancer field effects and the relationship between morphological and molecular features (Ščupáková, Dewez, Walch, Heeren, & Balluff, 2020). A MALDI-MSI-based scheme was proposed to chemically analyze the lipid and peptide content of a large number of hyaline and dense nuclear vesicles in single cells and to observe the chemical heterogeneity between them, and this method is now widely used to reveal the complex microenvironment within single cells (Castro, Xie, Rubakhin, Romanova, & Sweedler, 2021).

SIMS is the highest spatial resolution MSI technique available, which is label-free, with high sensitivity, multi-component detection, submicron level high spatial resolution imaging (Castro, Xie, Rubakhin, Romanova, & Sweedler, 2020). SIMS is not only suitable for elemental analysis, but also for MSI studies at single cell and even subcellular level due to its extremely high resolution (Cai, Xia, Li, Zhang, & Zhang, 2021). The nanoscale SIMS (NanoSIMS) showed high sensitivity and spatial resolution, which enabled defining the uptake and distribution of antisense oligonucleotides (ASO) in different subcellular compartments, providing new insights into the use of nucleic acid-based therapeutics for the treatment of human diseases (He et al., 2020). The gas cluster ion beam SIMS (GCIB-SIMS) was utilized to visualize the physiologically relevant levels of very low abundance lipid peroxide in subcellular compartments and its accumulation under disease conditions, providing a reference for localizing phospholipid iron body death signals in cells and tissues (Sparvero et al., 2021).

4. MSI can be applied to unearth TCM Q-marker

There is a wide variety of species and active ingredients that can act together through multiple pathways and targets, but there are still many mechanisms of action of TCM that have not been clearly elucidated. Currently, there are numerous analytical methods for the identification, analysis, and quality control of TCMs, e.g., thin-layer chromatography (TLC) (Deng, Jing, & Liu, 2019), GC–MS (Xu & Zhang, 2007), and LC-MS (Wu et al., 2016). The rational use of different analytical methods can provide a reliable basis for the discovery of active components of TCM and their related mechanisms of action (Liu, Zhang, Shan, Zhou, & Hou, 2012).

MS is considered to be the most information-rich technique and is widely used in herbal medicine research. However, the object of MS analysis is usually a homogenized sample (Zhang, Sun, & Wang, 2018), which can lead to the loss of spatial information of the analyte. In recent years, MSI techniques can be used to determine the molecular composition and spatial distribution of herbal medicines (Jiang, Zhang, Liu, Wang, He, & Jin, 2022), which represents a major breakthrough in the analysis of herbal medicines and helps understand the functions and regulatory pathways of specific components in herbal medicines. Although there are still many challenges and obstacles, such as difficulties in sample pretreatment due to the histomorphological diversity of TCMs, the specificity of the preparation method, and difficulties in achieving high spatial resolution imaging, many studies have made remarkable progress (Peng, Chen, & Zhou, 2020).

4.1. Detecting and tracking material changes during growth and development of TCMs plants

At present, MSI technology has been widely used in multidimensional and multi-level studies on monitoring the growth and development process of TCMs plants, changes in endogenous substance groups and their spatial distribution. For instance, the spatial distribution of endogenous molecules during fruit development of *Lycium barbarum* L. was visualized by MALDI-MSI, the distribution changes at different developmental stages were investigated, and the spatial distribution of endogenous molecules in *L. barbarum* fruit tissues was mapped, which provide an important scientific basis for exploring the changes and accumulation processes of endogenous substance groups in TCMs plants (Zhao, Zhang, & Shi, 2021). Meanwhile, Xia et al. applied DESI combined with metabolomics to explore the distribution of chemical components in different parts and different periods of *Ganoderma lucidum* (Leyss.ex Fr.) Karst, the changes of lipid content and the accumulation of triterpenoids in *G. lucidum* at different stages were found (Xia et al., 2024). Through these, we can clearly feel the advantages of MSI in TCMs research.

4.2. Identifying and characterizing TCMs species and components

The high-resolution matrix-assisted laser-resolved ionization MSI (MALDI-HRMS) was used to perform spatial chemical analysis of chrysin and its related chemicals, such as Kanga and Olympic chrysin, in plants of the genus *Chrysanthemum*, providing a basis for further detailed studies of chrysin biosynthesis (Kusari, Sezgin, Nigutova, Cellarova, & Spiteller, 2015). In the same way, Li et al. used the high quality resolution MALDI MSI and MALDI tandem MSI were conducted on two Paeoniaceae herbal medicines, peony bark (*Moutan*) and peony, to analyze their spatial metabolomic similarities and differences, and first time visualize for the major metabolites involved in the galactoside biosynthesis pathway. It confirms that the potential of MSI in the study of identifying and characterizing TCM species and components (Li, Ge, Liu, Hu, & Li, 2021).

4.3. Observing the distribution of metabolism of TCM components

MALDI-MSI and MS molecular networks were combined to characterize the metabolite components of *Ginkgo biloba* L., and for the first time dozens of specific metabolites in the wood tissues of *G. biloba* were visualized, which is a major breakthrough in the study of wood-based TCM (Kuo, Huang, & Hsu, 2019). While, MALDI-MSI was also used to map the concentration profiles of flavonoid glycosides and biflavonoids in *G. biloba* leaves, and a significant distribution of all these substances in thin slices of *G. biloba* leaves was revealed (Beck & Stengel, 2016). This is a great inspiration for observing the metabolic distribution of each component in TCMs.

4.4. Probing composition differences between different medicinal parts of TCMs and before and after concoction

The differential distribution of characteristic components of root, stem, and leaf tissues of *Salvia miltiorrhiza* Bge. was dissected by MALDI-MSI to obtain the differences in compound abundance and content in different parts of *S. miltiorrhiza*, and the spatial distribution patterns of nine tanshinones and nine phenolic acids were disclosed (Li et al., 2020a; Li et al., 2020b). In order to characterize the changes in the composition of TCMs after processing, Li et al. combined with multi-component characterization and non-targeted metabolomics, revealed the compositional changes of *Ligustri Lucidi Fructus* (Nvzhenzi in Chinese) after wine preparation by MSI, and also demonstrated that the successive clusters of drug components in different wine preparation time periods presented a clear transformation trajectory, which provided more convincing data for the study of TCMs concoction (Li et al., 2020a; Li et al., 2020b). Likewise, the *Aconiti Lateralis Radix Praeparata* (Fuzi in Chinese) also requires different processing methods before it can be used to treat diseases, to this end, the DESI-MSI method was applied to explore the differences between *Aconiti Lateralis Radix Praeparata* at different steaming time points, it gives great inspiration to the development and application of traditional Chinese medicine processing (Liu et al., 2022).

In summary, MSI technology has been widely used in the research process of growth, development, species identification, component metabolism and concoction application of TCMs, enabling its application in the discovery and confirmation of TCMs Q-Marker.

5. A proposed method for discovery of TCMs Q-Marker based on MSI

5.1. Spatial and temporal distribution of chemical groups in vivo based on MSI

The distribution of chemical groups is often analyzed by techniques such as capillary electrophoresis (Gao, Wang, Wang, & Deng, 2013; Sun, Wang, Yao, & Lv, 2017), HPLC-chemiluminescence coupling (HPLC-CL) (Ma & Li, 2016), MS (Guo, Chi, Wang, Xiu, & Yue, 2021), and LC-MS (He, Yang, & Gao, 2007), but homogenization of samples is a prerequisite in these methods, which can blur the spatial distribution of chemical groups *in vivo*. Instead, the spatial and temporal distribution of chemical groups can be observed in situ and visualized by MSI.

MSI is applied to the *in vivo* distribution study of chemical substance groups by selecting continuous slices to reflect the dynamic changes in the location of active ingredients. Due to the specificity of multi-component and multi-target of TCM, it is often difficult to determine the spatio-temporal distribution of drugs *in vivo* with a single homogenized sample, therefore, MSI is mostly applied to determine the spatio-temporal distribution of chemical substance groups in TCMs. For example, DESI-MSI technique was used to study the pharmacokinetics of *Ginseng* Construction-Nourishing Decoction, many components of the formula, such as terpenoids and flavonoids, were transported to the brain after oral administration. The concentrations of drug components in the brain were very low and could not be quantified, and MSI technique can clarify the distribution of complex drug components, which facilitates revealing the active ingredients of TCMs formula and its mechanism of action (Matsumoto et al., 2021). The distribution and localization of ephedrine in the lung were visualized by MALDI-MSI analysis (Matsumoto et al., 2017), the detection and visualization of ephedrine signals in lung sections of rats gavaged with ephedra soup verified the arrival of the studied components to the lung tissue, indicating that ephedrine was distributed in the subepithelial region of the lung including the smooth muscle of the bronchi (Matsumoto et al., 2017). MALDI-MSI revealed the effects of *ephedra* extract, *ephedra* polysaccharide, and total alkaloids of ephedra in myocardial infarction rats (Wu et al., 2019), which showed that appropriate doses of *Epilobium* alkaloids could reverse the abnormal alterations in the levels and distribution patterns of molecules related to cardiac energy metabolism, phospholipids, potassium ions, and glutamine, thus improving the abnormalities of myocardial energy metabolism and altering the levels and distribution patterns of phospholipids. These results laid the foundation for subsequent studies on the mechanism of action of *Epilobium* in improving myocardial infarction (Wu et al., 2019).

Various studies have shown that MSI can be applied to observe the spatio-temporal distribution of chemical substance groups of herbal medicines in various parts of the body such as brain, heart and lung, which is an important part of the discovery of TCMs Q-Marker.

5.2. Endogenous substance group study based on MSI

The endogenous substance group includes small molecule metabolites, neurotransmitters, hormones, peptides, proteins, nucleic acids, lipids, etc., all of which are present in human, animals, and plants or released from organisms during different biological processes such as oxidative stress or metabolic transformations. The endogenous substance group is important for obtaining essential information such as biological components, metabolic pathways, and organismal changes.

The analysis of the endogenous group of substances consists mainly in identifying and analyzing the actual concentration levels in organization, and even in studying the biochemical pathways in which they may act. However, the sample matrices used, such as whole blood, serum, urine, stool, saliva, and tissue, are usually too complex to be analyzed directly by analytical instruments and there may be serious interference during the determination process. Before performing measurements, samples need to be processed and prepared, including sample extraction, processing, and enrichment, a step that is often critical, while the determination of concentrations often requires internal standards. The application of MS and other spectral analyses often results in the absence of spatially resolved information of samples, which is a drawback for the study of endogenous substance groups. In recent years, with the aid of label-free and high sensitivity MSI, the study of endogenous substance groups has made great progress (Huang et al., 2019), especially in lipids, peptides, and proteins.

5.2.1. Lipids

Lipids are one of the important nutrients required by the human body. In order to explore the origin of kidney diseases from a lipid perspective, MALDI-MSI was applied to identify the histological structure of lipids in the kidney and analyze the differences in lipids that can be observed between different tissue regions, so as to elucidate the pathogenesis of atherosclerosis (Martín-Saiz et al., 2021). The MS-assisted imaging and other methods were combined to explore the origin and development of arterial lipids, which found that lipids accumulated to form a thickened inner membrane; such lipid pools form the lipid core of atherosclerosis, highlighting the important role of lipids in this disease and paving the way for appropriate treatment and prevention of atherosclerosis (Nakagawa et al., 2021). DESI-MSI was utilized to explore the spatial distribution of endogenous lipids in the skin, where the detection and determination of endogenous lipids in the skin can be useful in inferring the possible penetration pathways of the molecules, offering the prospect of further skin formulation studies (Quartier et al., 2021).

5.2.2. Proteins

The endogenous substances such as peptides and proteins can widely express the organism condition and the interaction between substances, and MSI techniques can be used to trace and analyze the peptides and proteins in the relevant biological processes, laying the foundation for their application in revealing the molecular roles, etc. For example, the nano-DESI approach was developed for MSI of proteins and protein complexes in rat kidney tissue to determine the spatial distribution of intact protein complexes and folded monomeric proteins (Hale & Cooper, 2021). The immuno-MSI (iMSI) was used to sensitively and reproducibly quantify anti-muscle atrophy proteins in mouse and human tissues, which helps understanding the fundamental processes in muscle biology regarding muscle fiber strength and stability, and may identify novel therapeutic targets and pathogenic mechanisms for the clinical treatment of muscle atrophy (Bishop et al., 2021). The nano-DESI of ligand-binding proteins and metal-binding proteins in rat brain identified a series of endogenous metal-binding protein complexes, as well as a large number of monomeric proteins with particularly different spatial distributions, offering great potential for biomedical translation and application (Sisley, Hale, Styles, & Cooper, 2022).

5.3. Finding Q-Marker from “chemical substance group – Endogenous substance group” of TCMs

In recent years, MSI technology has been widely used in the characterization of complex components of TCMs, such as xenobiotic chemical substance group and endogenous substance group, laying a foundation for the application of MS technology in the discovery of TCMs Q-Marker. In chemical substance group, MSI is applied to detect the spatial and temporal distribution of drug-

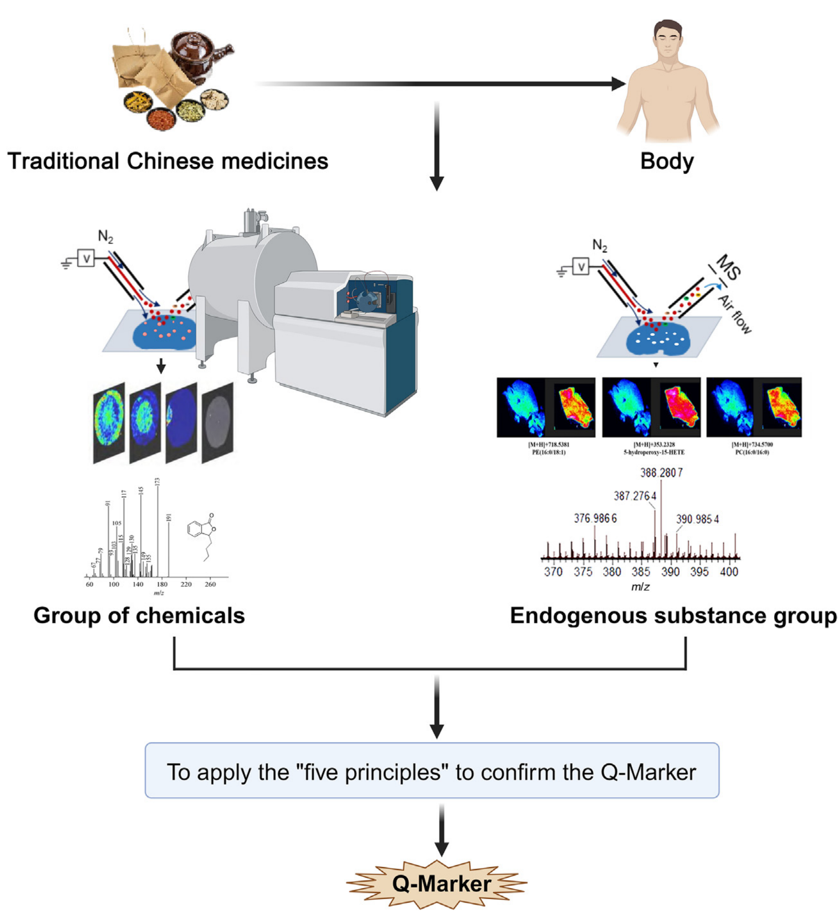

Fig. 3. Discovery of TCMs Q-Marker based on MSI technology. After drug is given to the body, we perform MSI for group of chemicals and endogenous substance group, respectively, and combine the “five principles” to confirm Q-Markers of TCMs.

related components, explore the enrichment site, spatial distribution and aging relationship of related components according to different mass charge ratio, which can simplify and normalize complex and diverse chemical components in TCMs. The target tissues of the respective components can be directly observed; according to the theory of nature and flavor/taste of TCMs, the results of drug actions can be verified, clarifying the chemical group of TCMs and facilitating the discovery of Q-Marker.

Unlike xenobiotic substance groups, the endogenous substance groups are naturally present in the body, which are able to extract water from cells, molecules, etc., and respond to changes in body physiology, diseases and drug effects. Under different environmental conditions, the changes and differences of endogenous substance groups need to be monitored in time to correctly grasp the real-time state of the body. MSI can be used to observe the changing trend of endogenous substance groups under different physiological states, which will provide new reference and ideas for the discovery of TCMs Q-Marker.

In the applications of MSI (Fig. 3), it is compelling to effectively associate "chemical group and endogenous substance group", dynamically observe the changing relationship between them (Jing, Liu, Deng, Chen, & Zhou, 2019; Chen et al., 2023), and investigate the role and effect of TCMs components in the body in a more comprehensive and multidimensional way, so as to better discover the Q-Marker.

6. Conclusion

For decades, TCMs researchers have conducted a lot of research and exploration on the quality evaluation and QC of TCMs, with great progress. The proposal of Q-Markers directly focused on the essential connotation of the quality attribute of TCMs, which highlights the effectiveness of TCMs drugs, measurability of components, transmission and traceability of quality and other aspects; the theories of TCMs and related disciplines should be integrated, and the true effective components of TCMs should be systematically analyzed, so as to achieve a new improvement in the quality control of TCMs.

At present, MSI has become the mainstream frontier technology for the analysis 1of histochemical imaging, single-cell and subcellular structure imaging of animals and plants. Compared with traditional medical imaging methods, such as positron emission tomography, nuclear magnetic resonance imaging and fluorescence imaging, MSI does not require complex biomarker processes. The complex time–space distribution information of multiple biomolecules in samples can be directly and simultaneously obtained, which undoubtedly provides great help for the analysis of multivariate changes of complex components.

The application of MSI in the discovery and confirmation of TCMs Q-Marker is based on the premise that MSI can disclose the substance composition of TCMs and the changes in the *in vivo* environment, and then Q-Marker can be found by summarizing the spatiotemporal changes of endogenous substance groups and chemical groups of TCMs. Finally, the candidate TCMs Q-Markers can be confirmed by the "five principles" of Q-Markers.

The discovery and confirmation of TCMs Q-Marker is a process that needs continuous exploration and improvement. With the rapid development of analysis technology and interdisciplinary integration, the Q-Marker research has made significant progress in all aspects. The addition of MSI will provide new feasibility for the discovery and confirmation of TCMs Q-Marker, by which the alteration rule between chemical substance groups and endogenous substance groups can be directly characterized, and the quantitative relationship and contribution between chemical substances of TCMs and the overall biological effect can be visually revealed. MSI will be a powerful research tool for TCMs Q-Markers and substantially contribute to the development of TCMs standardization and internationalization.

CRediT authorship contribution statement

Zhiyun Wang: Writing – original draft. **Huajie Chang:** Writing – review & editing. **Qian Zhao:** Writing – review & editing. **Wenfeng Gou:** Supervision. **Yiliang Li:** Conceptualization. **Zhengwei Tu:** Data curation, Project administration. **Wenbin Hou:** Writing – original draft.

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.

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