



## Research

## Traditional Chinese Medicine—Review

# Deeper Chemical Perceptions for Better Traditional Chinese Medicine Standards



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## ABSTRACT

Traditional Chinese medicines (TCMs), a complex system of natural resources with many diverse components, are widely used as approved medicinal agents in China. Quality control of TCMs is a huge challenge for the government and for testing institutes and is associated with numerous scientific issues. Among these considerations include the following questions: How many components are in TCMs? How can the multiple components in TCMs be comprehensively delineated and subsequently characterized? What is the level and range of these (active) metabolites within these multiple-component TCMs, in order to recommend standards? and What are the qualities required for a marker constituent to be selected, and from a practical perspective, how can these components be assessed with low cost and in a short time? All of these factors require significant and deep thinking in order to understand the individualistic chemistry of TCM in order to develop enhanced TCM quality standards for improved and consistent patient care. In this review, the latest exploratory research in TCM chemistry analytical techniques and methods is summarized in order to begin to develop responses to these scientific issues. Advances in these methods have included multidimensional separation for liquid chromatography–high-resolution mass spectrometry (LC–HRMS), smart triggering data-dependent acquisition of LC–HRMS, target analysis with liquid chromatography–mass spectrometry (LC–MS), supercritical fluid chromatography, and data mining of large mass spectrometry (MS) datasets. In addition, two quality strategies have been introduced in order to save reference standards and the analysis time for a TCM quality standard, including the application of the single standard to determine multi-components (SSDMC) and monomethod-heterotrait matrix methods. Finally, a series of future improvements for analytical methods for TCMs are proposed.

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## 1. Introduction

Traditional Chinese medicines (TCMs) have been used in China for several thousand years, and have played a pivotal role in maintaining the health of the Chinese people. With the global popularization of practices that seek a natural and holistic means of disease treatment, the demand for TCMs around the world has steadily increased. This has made the development of internationally accepted quality standards much more important than at any

previous time in human history [1]. The principal quality standards of drugs are reflected in the descriptions found in pharmacopeias. Nowadays, the *Chinese Pharmacopoeia* (ChP), the *United States Pharmacopoeia* (USP), and the *European Pharmacopoeia* (EP) are paying much more attention to increasing the amount and elevating the level of the monographs concerning plant-based drugs. As a reflection of this development in TCM globalization, 81 Chinese medicinal plants have been added to EP 9.2 and 37 Chinese botanical drugs and their preparations have been added to USP 41 [2].

A greater understanding of the chemical composition of TCMs could enhance holistic quality control, while the application of innovative quality control technologies now makes it more feasible to meet those challenges. Although a total of 618 Chinese crude

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drugs and decoction slices were recorded in the 2015 edition of ChP [3], these monographs often still fall short of reproducible holistic quality standards, and some even lack defined quantitative markers. It can be seen, therefore, that common quality control model is not suitable for the quality assessment of complex TCMs. Thus, we put forward a systematic quality research approach for TCMs, which moves from sophisticated and comprehensive research to the development of generally applicable simplified standards [4]. For this approach, a deeper perception of the chemistry of TCMs was the most important aspect for understanding and translating TCM standards. That is because the chemical constituents are the therapeutic foundation of TCMs, and thus have an inexorably strong link to TCM quality. Greater knowledge about chemical constituents profiles should also throw light on the four challenges of TCM quality research and the elaboration of quality standards: ① the analysis and characterization of the chemical components in TCM plants, especially those in TCMs comprised of compound formulas; ② the fact that the single-marker approach for quality control is not suitable for TCMs with a multiple-component system, making the development of a comprehensive quality control model an urgent need; ③ the need for clarification of the active or even effective components in TCMs; and ④ the need for elaboration of scientific, practical, and feasible quality standards that can be widely practiced [4]. One example is Chinese salvia (Danshen), which contains two main types of chemical constituents: the tanshinones, which were first discovered in the 1930s [5], and the salvianolic acids, which were isolated in the 1980s based on progress in developing separation technologies [6]. Thus, tanshinone IIA was first introduced as an identification marker in the ChP 1990, while salvianolic acid B was chosen as a quantified marker only in ChP 2005. With a better understanding of the chemistry of Chinese salvia and the progress of quality control methods, two more tanshinones—cryptotanshinone and tanshinone I—were simultaneously quantified with tanshinone IIA in order to improve holistic quality control, while maintaining the overall costs for testing [7]. The new monograph of Chinese salvia has now been recorded in USP 38 [8] and in ChP 2015 [3]. Recently, a new Q-marker concept was proposed that would change the paradigm of the TCM quality control pattern. It was defined as a natural, analyzable, functional, and traceable chemical component(s) from a TCM. This concept of a marker also displays the importance of a deep understanding of the chemical components of an individual TCM [9].

Liquid chromatography (LC)-mass spectrometry (MS) has been used for the detailed analysis of the chemical constituents of TCMs since 1997. The constituents of a single TCM generally include thousands of primary metabolites (polysaccharides, lipids, etc.) and secondary metabolites (alkaloids, coumarins, triterpenoids, flavonoids, lignans, acetogenins, etc.) [10]. In most cases, analytical studies have focused on the secondary metabolites—the small molecules with molecular weights below 2000 Da. Traditional phytochemical techniques, including chromatographic separation and structure elucidation, are still gold-standard operations to obtain precise information about the complexity of the chemical constituents in a TCM [11]. However, for a rapid understanding of the chemistry of a TCM, isolation procedures are too tedious and time-consuming. The invention of hyphenated analytical techniques that combine MS with a chromatographic technique provides an extremely powerful tool that can scrutinize trace components in a TCM, and can offer a bird's eye view of the chemical complexity of a TCM in a single run. In this respect, ultra performance liquid chromatography (UPLC)-MS and supercritical fluid chromatography (SFC)-MS are very powerful tools. Compared with a simple chromatographic instrument, such hyphenated instruments inform the scientist as to how many components are present in a TCM, and can reveal which of the constituents might

provide an exact mass-to-charge ratio ( $m/z$ ), regular ion fragment information, and ion's collision cross-section (CCS). With great progress in LC-MS during the past five years, it has become possible to smartly explore thousands of chemical components and quantify hundreds of analytes in a TCM. Such analytical power has opened a fascinating new era for perceiving the depth of the chemistry of a TCM, and has initiated the smart big data era for structure interpretation. It should be mentioned that the exploration of the large number of chemical components should be correlated with the overall quality and/or with functional activity, and should provide useful information for proposed TCM quality standards through smart data mining.

With a deeper understanding of the vast complexity of a TCM, the multi-components assay has been the consensus of TCM scientists. However, two problems hinder the application of multi-components quality control in general industrial practice. One is the high cost of the standard compounds, while the other is the tedious operation for the control of the same components in different TCM formulas. The single standard to determine multi-components (SSDMC) method was proposed to resolve the first problem [7], and this will now be widely adopted in ChP 2020. To address the latter problem, we proposed a monomethod-heterotrait matrix method that could be used for multi-component qualification and quantification in different TCM formulas using the same sample preparation and analysis method [12].

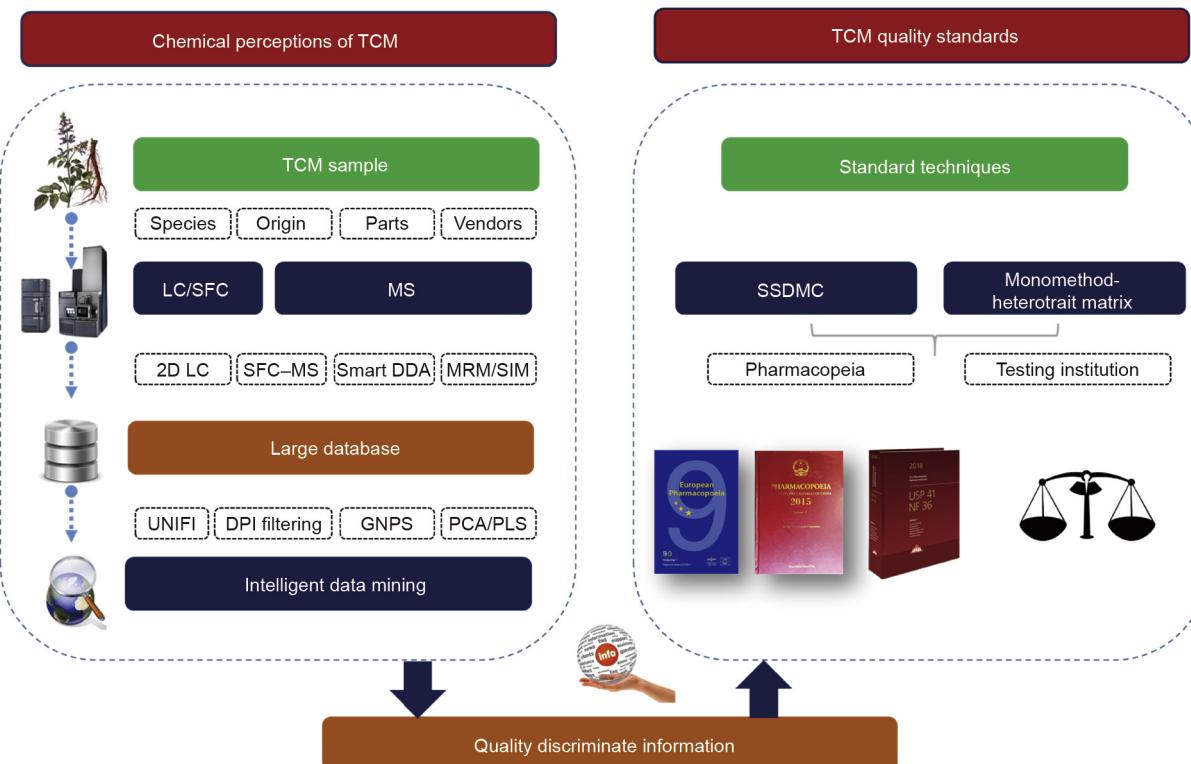
In 2009, we reviewed the application of analytical methods for the different secondary metabolites of TCMs [10]. The *in vitro* and *in vivo* application of MS in herbal medicine has been reviewed by Zhang et al. [13], and the bibliometric evaluation of LC-MS data for the analysis of TCMs from 1997 to 2005 has been reported by He et al. [14]. This review mainly covers the development in the last five years of new techniques that can deeply explore the chemical components of TCMs, including two-dimensional (2D) LC-MS, instrument-dependent data acquisition methods, instrument-independent data acquisition methods, multi-target quantified assay by LC-MS, new applications of SFC-MS, and smart data-mining methods. Two new quality control methods, including the SSDMC method and the monomethod-heterotrait matrix method were introduced in this period to establish improved TCM quality standards (Fig. 1).

## 2. Ideas for a deeper understanding of TCM chemistry and quality standards

### 2.1. Ideas for a deeper understanding of TCM chemistry

A holographic depiction of a TCM by a substance information database was the basic idea for developing a deep understanding of the chemistry of a TCM. As mentioned above, a TCM typically comprises thousands of chemical components. These contain all of the information about both the quality and functional activity of that TCM. Thus, the first important aspects to explore are the following three questions: ① How many chemical components are there in a TCM? ② What are the structures of these chemical components? and ③ What is the content level of these chemical components? However, even though these questions are relatively simple, it is still not possible to fully answer them using modern analytical technologies for some types of metabolites, such as polysaccharides, tannins, and so forth.

Fortunately, for the major secondary metabolites, which are widely regarded as the primary active substances, such as the coumarins, saponins, alkaloids, and flavonoids, many creative techniques have been reported and can better respond to the challenges of these three questions. Multidimensional LC-MS is



**Fig. 1.** Deeper chemical perceptions of TCM, for better TCM quality standards. Smart DDA: smart triggering data-dependent acquisition; MRM: multiple reaction monitoring; SIM: selective ion monitoring; DPI: diagnostic product ion filtering; GNPS: Global Natural Product Social Molecular Networking; PCA: principal components analysis; PLS: partial least squares regression.

the most important technology at present for exploring the diverse and complex chemical components in a TCM, including offline and online comprehensive 2D LC-MS analysis. When combined with ion mobility (IM) as a parameter, a four-dimensional analytical approach can be realized. The theoretical capacity of this approach to explore the chemical components of a 2D LC-MS can reach about 9000, which could fully meet the complexity of TCM [15]. All of the constituents obtained without ion fragment information are difficult to characterize and identify. Thus, many smart triggering data-dependent acquisition (smart DDA) methods to obtain MS/MS or  $MS^n$  fragment information have been proposed, including instrument-dependent methods and data post-processing methods. The SFC-MS method, which offers an orthogonal selectivity compared with LC-MS, is preferable for the exploration of lipids and isomeric components. For quantitative information on the explored chemical constituents, the widely targeted quantitative with multiple reaction monitoring (MRM) method has been developed. With the accumulation of chemical components datasets, smart data-mining methods should be adopted, such as auto MS data analysis using the UNIFI software, ion fragment MS/MS spectrum auto cluster using Global Natural Product Social Molecular Networking (GNPS), quality correlation and prediction using support vector mashing (SVM) or neural network (NN) techniques, and so on.

## 2.2. Ideas for TCM quality standards

The cost and efficiency of TCM quality standards testing should be seriously considered. For holistic quality standards, the cost of standard compounds is one of the primary obstacles for implementing the monograph, especially in a corporate setting. The SSDMC approach proposes to only use a single standard compound to accurately determine the content of all the other analytes; this

greatly decreases the demand for standard compounds. The long test time is the other factor making the test tedious, especially for qualitative or quantitative analysis of the same components in different compound formula TCMs. The monomethod-heterotrait matrix method for qualitative analysis is based on UPLC-QDa MS, which is a low-cost and compact single-quadrupole MS. In this technique, the characteristic markers of a plant medicine are monitored using a selective ion monitoring (SIM) method; then all of the Chinese patent medicines (CPMs) that involve the same herb can use the same method for qualitative control. For quantitative analysis, the 2D LC multi-heart-cutting (MHC) separation technology is adopted. The multiple analytes of a medicinal plant can be transferred in an orderly manner into a second column, which will minimize the interference of the matrix and be directly detected by means of an ultraviolet (UV) detector. This method could also be applied to all CPMs containing the identical herb.

## 3. Technological advances in perceiving TCM chemistry

### 3.1. Multidimensional separation for LC-MS

LC-MS has proved to be an exceptionally good tool for revealing the chemical basis, metabolism, and mechanisms of action for TCMs [16,17]. Despite improvements in configurations, columns, and detector sensitivities, due to the complexity of TCMs, conventional LC-MS methods still suffer from the possible co-elution of metabolites, especially between trace analytes and high-abundance analytes [18]. Strategies have been proposed to improve the performance, particularly in the aspects of chromatographic and mass separations, by adding an extra dimension of separation—namely, 2D LC and IM-MS.

Two-dimensional LC can be broadly divided into offline 2D LC and online 2D LC, according to the existence of the interface between the two dimensions. In offline 2D LC, the fractions after the 1D separation are collected, processed, and reinjected into the second dimension; this procedure is low in automation, since no interface is introduced [19]. For online 2D LC analysis, a special interface is introduced, and the fractions are transferred to the second dimension automatically. Depending on whether all the 1D eluents are subjected to 2D separations, 2D LC can be classified into comprehensive 2D LC and heart-cutting 2D LC [20]. In heart-cutting 2D LC, only those components of current interest are transferred to the second dimension.

The application of 2D LC in the analysis of TCMs was reviewed in 2014 [21]. In recent years, further improvements in 2D LC–MS have been realized, which has increased our ability to deeply comprehend the vastness of TCM chemistry.

### 3.1.1. Offline 2D LC

Offline 2D LC is free from solvent-incompatibility issues, as the fractions from the first dimension are concentrated and redissolved before being injected into the second dimension. Thus, the system renders orthogonal hyphenations of different separation mechanisms and provides remarkably augmented peak capacity [15,22]. Although it suffers from a loss of sample, low efficiency, low automation, and possible contamination, due to the human interventions of the concentration, redissolution, and reinjection procedures [21], it is still the most powerful mode focused on the comprehensive profiling of complex TCMs.

In general, there are four combination modes to develop offline 2D LC–MS systems, including normal phase (NP) × reverse phase (RP), hydrophilic interaction liquid chromatography (HILIC) × RP, RP × RP, and cell membrane chromatography (CMC) × RP. RP columns are often chosen as the second dimensional columns in all of these modes, since they have been observed to have high peak capacity and good compatibility with MS.

(1) **NP × RP.** The NP column is a good alternative to the RP column due to its distinct retention mechanisms. Toad skin is one of the most famous TCMs with strong antitumor activities. Zhang et al. [23] have developed a comprehensive offline 2D NP/RP LC–MS method to detect the bufadienolides, based on an XAmide column in the first dimension and an XUnion C<sub>18</sub> column in the second dimension. The orthogonality of the system was investigated with 15 bufadienolide mixtures by the geometric approach, and the result was 49.6%. Finally, 64 bufadienolides including 33 minor ones and 11 pairs of isomers were identified in toad skin. The same group also developed two offline 2D LC methods in order to quickly prepare the bufadienolides based on HILIC × RP and RP × RP systems [24,25].

(2) **HILIC × RP.** Although the NP shows high orthogonality with RP columns, due to its less environmentally friendly solvents and limited range of applications, it has not been widely utilized, even in 2D LC systems. HILIC has often been considered to be “highly aqueous NP” chromatography, with acetonitrile and water as the main mobile phase. Unlike RP, water is the stronger eluting solvent. In 2016, Jin et al. [26] reviewed the recent developments and applications in HILIC stationary phases. HILIC showed complementary separation with conventional RP stationary phases for glycosides, oligosaccharides, steroids, and phenolic acids. The key point of the mode is the order in which the HILIC should be used as the first dimensional column. This model was successfully used on ginsenosides of the genus *Panax* (P.) [15,27,28]; quinonochalcone C-glycosides and flavonoid O-glycosides of *Carthamus tinctorius* (C. *tinctorius*) (flos, Chinese name: Honghua) [22,29]; the phenolic acids of *Salvia miltiorrhiza* (rhizome, Chinese name: Danshen); and components from *Ginkgo biloba* extract [19,30]. To characterize the ginsenosides from the stems and leaves of *P. ginseng*, a 2D LC sys-

tem combined with an XBridge Amide column (1D) and an ethylene bridged hybrid (BEH)-C<sub>18</sub> column (2D), coupled with a linear ion-trap quadrupole (LTQ)-Orbitrap system, was developed. The orthogonality of the offline 2D LC system was 69%, and the peak capacity could reach 8925. A total of 646 ginsenosides were characterized with 427 potentially new metabolites, in comparison with only 289 saponins having been reported in 11 *Panax* species before 2012 [31]. In 2018, 945 ginsenosides from the leaves of *P. notoginseng* (rhizome, Chinese name: Sanqi) were first screened with the same 2D LC system combined with new data-dependent acquisition (DDA), revealing 662 potentially new ginsenosides (Fig. 2) [15]. For this mode, the analytes were moderately polar, and could be retained in both HILIC and RP.

(3) **RP × RP.** A pH orthogonality was first applied using this mode. It was composed of a positively charged RP column (Acchrom XCharge C<sub>18</sub>) using an acidic condition mobile phase (1D) and a conventional RP column (EVO C<sub>18</sub>) in the basic mobile phase (2D). Indole alkaloids from five botanical origins of Gouteng (*Uncaria*) recorded in ChP 2015 were first screened by this mode. The orthogonality of the offline 2D LC system was 74%. A total of 1227 indole alkaloids were efficiently shown to be present and characterized from five botanical origins of Gouteng, which showed the high chemical diversity of the species [32]. For this mode, alkaloids were more suitable.

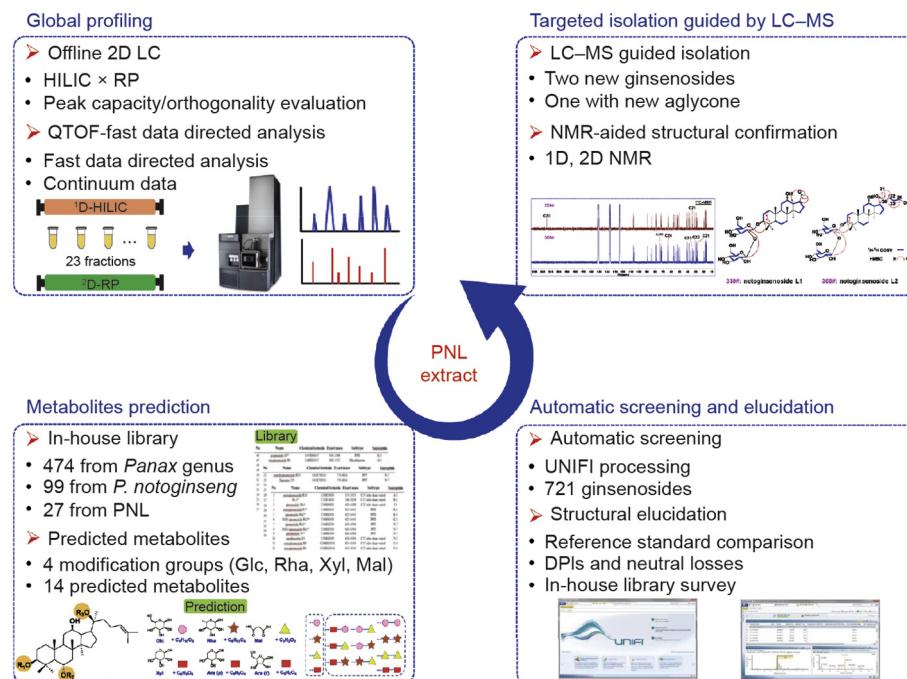
(4) **CMC × RP.** CMC is a type of bioaffinity chromatography. Cell membranes with specific receptors are adsorbed on the surface of activated silica to form a cell membrane stationary phase. Only components with high affinity for the receptor are retained [33]. Construction of a 2D LC system with CMC as the first dimension may help to overcome the shortcomings of short column life, low column efficiency, low peak capacity, and low efficiency in structure identification. Yue et al. [34] analyzed the extract of *Coptis chinensis* (rhizome, Chinese name: Huanglian) using a self-prepared beta-1 adrenergic receptor (β<sub>1</sub>AR)/CMC column; the fractions were further analyzed by means of UPLC–MS. Finally, coptisine was identified as the main active component that could inhibit β<sub>1</sub>AR. This result was further confirmed by the pharmacological test *in vitro*. For this mode, potent activity components could be screened out directly.

### 3.1.2. Online 2D LC

For online 2D LC analysis, a special interface was introduced, including switching valve(s) and loop(s), and the fractions being transferred automatically into the second dimension column. Comprehensive online 2D LC–MS analysis of TCMs generally has a long 1D separation time (1–2 h, to improve the resolution) and short 2D separation time (~30 s, limited by the volume of the bypass sampling loop). Thus, its peak capacity is generally smaller than that of offline 2D LC–MS; however, its repeatability is much better than offline 2D LC–MS, and it is more suitable for the analysis of multiple samples.

(1) **Innovations at the interface.** For the comprehensive online 2D LC system, the interface is the most important aspect in order to transfer all of the fractions automatically from the first to the second dimension. The HILIC × RP system also showed good orthogonality in the online 2D LC system; however, it was necessary to dilute the 1D eluate prior to injection into the 2D column in order to eliminate the solvent effect. For the analysis of the phenolic acids in Chinese salvia, a back-flush trap column was selected as the optimum interface, including an adjustable flow splitter, 100 μL sample loop, and 100 μL solvent mixer. A total of 196 peaks were successfully separated and detected in Chinese salvia. The proposed system exhibited orthogonality as high as 73% [35].

(2) **Innovations in the column and mobile phase.** Orthogonality and the organic phase strength of the 1D column were the primary considerations for the online 2D LC–MS. Zhou et al. [36]



**Fig. 2.** A typical flowchart for global profiling with offline 2D LC-high-resolution mass spectrometry (HRMS) and predicted metabolites data-matching-based UNIFI software from *P. notoginseng* leaves (PNL). First, an orthogonal HILIC × RP LC-quadrupole time-of-flight (QTOF) MS system was applied to characterize the ginsenosides in PNL. Second, in-house libraries of the ginsenosides from *Panax* genus, *P. notoginseng*, and PNL were constructed, and 14 predicted metabolites were generated for each item in the library of *P. notoginseng*. Third, the ginsenosides were then automatically screened out by predicted metabolites screening (PMS) in UNIFI with the predicted library, and the interpretations were conducted by fragmentation behavior studies of reference-standard ginsenosides. Finally, two compounds were purified by LC-MS guided isolation, and their structures were unambiguously identified by nuclear magnetic resonance (NMR) spectroscopy for validation. Glc: glucose; Rha: rhamnose; Xyl: xylose; Mal: malonyl. Reproduced from Ref. [15] with permission of Elsevier B.V., © 2018.

proposed a newly developed phenyl/tetrazole sulfoether (PTAS) bonded stationary phase, which was introduced in order to construct an RP × RP 2D LC system. The PTAS column (1D, 2.1 mm × 150 mm, 5 µm) had a very different selectivity from the BEH-C<sub>18</sub> column (2D, 3 mm × 50 mm, 1.7 µm), in that the orthogonality could reach 93.2%, and the remaining weaker hydrophobic property of PTAS made it very compatible with the C<sub>18</sub> column. The system was used for the analysis of *Curcuma kwangsiensis* (rhizome, Chinese name: Ezhu), in which 439 peaks (from the positive and negative ion modes) were counted, and 105 compounds were grouped and tentatively identified, including 73 previously unreported metabolites. A mixed-mode stationary phase also has the advantage of improving the orthogonality. The improved SAX-CN × C<sub>18</sub> system showed a better peak distribution and a reasonable analysis time compared with the SAX-PFP × C<sub>18</sub> system for the analysis of *Hedyotis diffusa* Willd (herba, Chinese name: Baihua sheshacao) and *Scutellaria barbata* (herba, Chinese name: Banzhilian) [37]. In addition, the CMC column could be used as a 1D column in an online 2D LC-MS system. It was used successfully to discover 16 potential active alkaloid components that counteract doxorubicin (DOX)-induced heart failure from *Aconitum carmichaelii* (radix, Chinese name: Fuzi), and three potential anti-hepatoma components—wogonin, oroxylin A, and neobaicalein—from the drug-containing serum of rats after oral administration of *Scutellaria baicalensis* Georgi (radix, Chinese name: Huangqin) [38,39].

For comprehensive online 2D LC, the use of very low flow rates in 1D separation will maintain the transfer volume at a minimum and increase the sampling from each 1D-separated peak that could be cut more times into the 2D separation. Thus, to profile the secondary metabolites of licorice, a microbore SeQuant ZIC-HILIC column (1 mm × 50 mm, 3.5 µm) was used in the first dimension in order to obtain slow separations in the first dimension and the

minimum transfer volume with decreased elution strength. As a result, 89 components were detected and, interestingly, licorice samples from different geographical locations could be differentiated using the detected specific compounds [40]. For the RP × RP 2D LC system, organic solvents in the mobile phase could also be optimized to improve orthogonality. In the analysis of phenolic compounds and triterpenoid saponins in licorice, a synchronized gradient mode was used to improve the chromatographic resolution and a total of 311 compounds were detected within 40 min. The peak capacity was 1329, and the orthogonality was 79.8% [41]. Similarly, 72 triterpenoid saponins in *Gleditsia sinensis* (fructus abnormalis, Chinese name: Zhuyazao) were separated and characterized using a comprehensive RP × RP 2D LC-MS system [42].

(3) **Combining with the MHC technique.** Due to the complexity of TCMs, and especially of compound TCM formulas, some elutes from 1D separation should be further separated using an MHC technique after comprehensive 2D LC-MS. The MHC conducts the collection, preservation, and analysis of consecutive fractions through an array of loops that allows for a 2D separation time of up to 3–5 min. Using this technique, many more components can be discovered after comprehensive 2D LC. It was used for the analysis of two CPMs (Gegen-Qinglian decoction (GQD) [43] and Dengzhan Shengmai capsules (DZS) [44]). For GQD, the same column configuration as the comprehensive 2D LC was used, and the 1D eluate at 4.4 min was loaded into 11 40-microlitre loops. These 11 fractions were successively separated by 2D separation to resolve 13 additional compounds [43]. For DZS, a different chiral high-performance liquid chromatography (HPLC) column was used as the 2D column of MHC, and an additional 12 pairs of isomers were separated with good resolution [44]. This technique can also be used to remove the major compounds in order to explore the presence of more minor components. Using this mode, a total of

271 and 254 peaks were separated from extracts of *Pueraria lobata* (radix, Chinese name: Gegen) and *Pueraria thomsonii* (radix, Chinese name: Fenge), respectively, within 35 min [45]. This technique might also be used to resolve the difficulties in multi-components assays in CPM.

### 3.1.3. Ion mobility

An IM spectrometer separates ions in the inert gas phase through an IM cell under the influence of an electric field [46]. The drift time of the ions, a key parameter for the determination of the CCS, is based on their size, shape, and charge. CCS is a physical property for a given compound in a specific condition [47]. Therefore, it may be a vital parameter for library construction [48,49]. The different time scales of chromatographic separation (seconds), IM (microseconds), and mass detection (milliseconds) allow them to work in series. When they were coupled, one more dimension other than LC–MS analysis was added, which showed advantages for the separation of isomeric compounds in complex systems, including TCM [50], metabolomics [47], and proteomics [51].

Tose et al. [52] separated the isomers of cannabinoids from marijuana, hashish, and the flowers and leaves of *Cannabis sativa* plants by UPLC combined with IM–MS. The CCSs of the isomers of cannabinoids were calculated with a modified version of MOB-CAL and associated with traveling-wave ion-mobility mass spectrometry (TWIM-MS) results. Pacini et al. [53] analyzed the pigments in microalgae samples using ultra-high performance liquid chromatography (UHPLC)-UV-TWIM-MS. The pigments could be highlighted by a characteristic absorbance at 450 nm, and 31 different pigments were further resolved and identified by TWIM-MS, while just 26 pigments were found using only UHPLC-UV-MS. These characterization results may also help to differentiate the microalgae species of *Chlorella vulgaris*, *Dunaliella salina*, and *Phaeodactylum tricornutum*. Wang et al. [50] achieved the separation of the isomers of crocin-3 and crocin-4 with IM–MS in the analysis of the fruits of *Gardenia jasminoides* (Chinese name: Zhizi). Willems et al. [54] differentiated the caffeoylquinic acids based on the intensity for the major product ions, and separated them by electrospray ionization (ESI)-high-field asymmetric-waveform ion-mobility spectrometry (FAIMS)-MS. A new method taking less than 1 min was adopted to separate and identify the mono-caffeoylequinic acids in apple/pear juice samples. Zhang et al. [55] established a multistage MS approach to provide a more reliable fragmentation relationship between precursor and daughter ions by integrating in-source collision-induced dissociation (ISCID) and time-aligned parallel fragmentation, and then applied the approach to study the fragmentation behavior of polycyclic polyphenylated acylphloroglucinols (PPAPs). A total of 140 PPAPs were detected from the crude extract of *Garcinia oblongifolia* (fructus, Chinese name: Shanzhuzi), with seven pairs of co-eluting, iso-baric PPAPs differentiated specifically by UHPLC–IM–MS. A 2D LC system was coupled to IM–MS to construct a four-dimensional separation system, and then applied to analyze the plant extracts from *Ginkgo biloba*, *Hedyotis diffusa*, and *Scutellaria barbata*. Each compound delivered only one peak in the second dimension, with a log modulation time of 4 min [56,57]. With IM assistance, the same *m/z* ions might be differentiated by their different molecular shape on high-resolution mass spectrometry (HRMS), which is impossible without IM.

### 3.2. Smart DDA of LC–HRMS

Multidimensional separation could expose as many TCM chemical components as possible; however, without accurate *m/z* and ion fragment information, most of them will not be identified. With the extensive use of HRMS, the acquisition of ion fragments

becomes the most important aspect. Ion fragment information can be obtained by DDA and data-independent acquisition (DIA). Some reviews have discussed the two modes [58,59], but most of the studies about screening components from TCMs were designed to smartly acquire ion fragments based on the DDA mode. The more target ion fragments are obtained, the more chemical components of TCMs can be deciphered. Analysis of the minor components based on characteristic fragment information using LC–MS has recently been reviewed [60,61]; here, we summarize the new DDA strategy for obtaining more fragments.

DDA can be triggered based on ion intensity, mass inclusion list or exclusion list, isotope pattern, pseudo neutral loss (NL), and mass defect. For deep profiling of the type of constituents in TCMs, smart DDA was designed; this can be divided into the instrument-dependent and instrument-independent acquisition.

#### 3.2.1. Instrument-dependent smart DDA

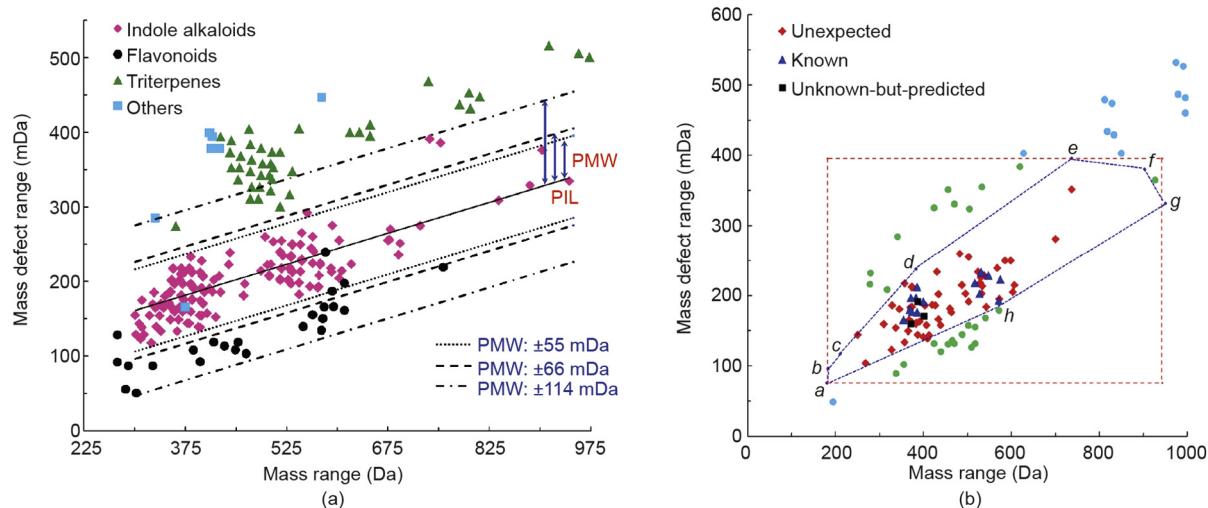
Most instrument-dependent smart DDA were developed on trap-mode instruments, such as LTQ-Orbitrap system or ion trap (IT)-time-of-flight (TOF) system. Three recent methods have been proposed, including the NL-triggered MS3 (NL-MS3) method, the mass tag-triggered DDA, and the step-wise precursor ion list (PIL)-based raster-mass defect filtering (MDF)-triggered DDA.

NL-MS3 is the method that has been used most often. This method has been used to identify specific classes of compounds sharing the same residues or conjugates, such as flavonoid O-glycosides [22], malonyl conjugates [62], and fatty acids conjugates [63]. NL-MS3 was designed to confirm dicarboxylic acid conjugated bufotoxin (DACB) with a special NL list that was obtained by screening various side chains (dicarboxylic acid) under high-energy C-trap dissociation (HCD). In all, 78 DACBs were discovered in *Venenum bufo* (Chinese name: Chansu), of which 68 were potential new compounds [63].

Mass tag-triggered DDA has been used to explore the malonyl-ginsenosides in ginseng extracts. By utilizing the fragment rule of malonyl-ginsenosides, in which the negative-mode collision-induced dissociation (CID) of malonyl-ginsenosides is prone to eliminating CO<sub>2</sub> (43.9898 Da), ISCID was adopted. Then, by enabling the “mass tag” function, an LTQ-Orbitrap mass spectrometer was used to determine the *m/z* values difference in MS1. If a mass pair consistent with the defined mass tag(s) exists in the full-scan spectrum (with an ion intensity higher than the threshold), the instrument turns off the ISCID energy and triggers multistage activation MS/MS of the selected ion pairs. In this way, only components with an ion fragment equal to 43.9898 Da in MS1 can obtain MS2. Next, an NL (43.9898 Da)-MS3 was integrated to confirm the malonyl-ginsenoside. This strategy was much smarter and avoided complex data processing [64].

Step-wise PIL-based raster-MDF-triggered DDA was developed to screen the indole alkaloids in five botanical origins of *Uncariae Ramulus cum Uncis* (Chinese name: Gouteng) (Fig. 3(a)) [32]. A step-wise PIL of theoretical indole alkaloids was created by means of a line equation and an optimal parent mass width (PMW) parameter in the LTQ-Orbitrap. The equation was established using integer mass as the x axis and mass defect as the y axis, based on the in-house *Uncaria* alkaloids library. The PMW was optimized to cover potential indole alkaloids and exclude other components as much as possible. This method was very convenient, compared with the traditional MDF method, and permitted more potential new alkaloids to be identified.

For these three smart DDA methods, the NL-MS3 is suitable for glycoside-type or ester-type components, whereas the mass tag-triggered DDA is only suitable for components with a loss of fixed mass at ISCID. For the last DDA mode, suitable components should have a linear relationship between the MDF and the mass within a narrow fluctuation range.



**Fig. 3.** Illustration of the development of (a) a step-wise PIL-based raster-MDF scan and (b) the polygonal MDF used in screening indole alkaloids from *Uncaria*. Eight vertexes of the polygon were indicated with a, b, c, d, e, f, g, and h. PMW: parent mass width. (a) Reproduced from Ref. [32] with permission of Elsevier B.V., ©2018; (b) reproduced from Ref. [69] with permission of Elsevier B.V., ©2017.

### 3.2.2. Instrument-independent smart DDA

PIL-triggered DDA is often instrument-independent, which means that they can be used on most high-resolution tandem mass spectrometry instruments. To smartly produce PIL containing a specific type of component, three novel methods, including polygonal MDF, in-source multiple collision (IMC)-neutral loss filtering (NLF), and fine isotopic pattern filtering (FIPF), have been put forward.

MDF is generally realized based on software, such as AB Sciex PeakView [65], Waters MetaboLynx XS [66], Thermo Scientific MetWorks [67], and Microsoft Excel [68]. Different algorithms containing classical rectangular MDF and modified MDF have been successively designed to find an acceptable compromise between decreasing false positives and expanding target coverage [12,23]. MDF has been used to selectively detect, extract, or analyze the components of interest using HRMS data. Different types of chemical components of TCM can be effectively discriminated by MDF. Two polygonal MDF algorithms [68,69] were developed, which differed from the common rectangular plotting algorithm. For the screening of the saponins in *P. notoginseng*, a pentagon plotting algorithm was designed to pick out the precursor ions of interest. For the screening of the indole alkaloids from *Uncaria sinensis* (ramulus cum uncis, Chinese name: Huagouteng), a polygonal MDF algorithm (Fig. 3(b)) was developed [69]. This was based on an in-house *Uncaria* alkaloids library and on molecular design. All of the potential indole alkaloids in *Uncaria* were covered by the mass range–mass defect range (mass range refers to the integer part of an observed  $m/z$ ) and exhibited an octagonal region depicted by eight vertexes; the PIL could then be generated, based on the limitation from the total ion list of the full MS, which was used to trigger DDA in order to confirm the types of indole alkaloids.

IMC-NLF was used to produce a PIL of malonyl-ginsenosides in *P. ginseng*. This was designed by utilizing the fragment rule of malonyl-ginsenosides, which is to fragment at low energy to produce  $\text{CO}_2$  or malonyl. First, multiple source collision energies were used to obtain full-scan raw data, which was processed and exported into the comma-separated value (CSV) table. Next, NL-MS finder software was used to produce the PIL. A total of 69 malonyl-ginsenosides from *P. ginseng* were obtained and confirmed [62].

FIPF was used to screen sulfur derivatives in TCMs with the resolution set at 100 000 full width at half maximum (FWHM) at  $m/z$

= 400. Fumigating TCMs with sulfur is a major issue, as it can produce new sulfur derivatives with unknown biological effects. However, it was difficult to find new sulfur derivatives with traditional isotopic pattern filtering (IPF) because the natural stable  $^{34}\text{S}$  is not as abundant as  $^{37}\text{Cl}$  and  $^{81}\text{Br}$ , which means that the  $^{34}\text{S}$  signal will be drastically influenced by the contribution of  $^{13}\text{C}_2 + ^{18}\text{O}$ . According to the  $M + 2$  isotopic compositions ( $^{12}\text{C}_x\text{H}_y\text{O}_z\text{S}^{32}\text{S}^{13}\text{C}_2\text{O}^{18}\text{O}$ ,  $^{12}\text{C}_{x+2}\text{H}_y\text{O}_z\text{S}^{34}\text{S}$ ), the  $M + 2$  of sulfur derivatives differed by a  $\Delta m$  of 0.0098 Da. With this finer criteria, nine sulfur derivatives from *Pueraria lobata* (Gegen) and *Pueraria thomsonii* (Fenge) were tentatively identified, and 55 commercial samples were rapidly compared, showing that the process of sulfur fumigation could be identified in more *Pueraria lobata* samples [70].

Of the three methods described above, polygonal MDF is suitable for a sample with most of its components being of the same type. The IMC-NLF method can be used for a great deal of raw data obtained with DIA. Regarding FIPF, only components with a special element can be used. Most PIL-triggered DDA methods will combine with dynamic exclusion (DE), as was shown in the analyses of *Pueraria lobata* (Gegen) [71], the leaves of *Citrus reticulata* (Chinese name: Chenpiye) [72], and Xiangsha-LiuJunzi-Jiajian granules [73]. It should be pointed out that a long list of masses all over the MS run might lead to a slow duty cycle and to data loss, and that the parent ion list can be separated into two or more lists. Shen et al. [74] characterized 190 polymeric phenolic acids in *Salvia miltiorrhiza* (Danshen) using two separated lists with around 250 compounds in each.

### 3.3. Target analysis with LC-MS

Multidimensional separation with LC-MS is often used for the untargeted analysis of chemical components. However, the target analysis is also important, as it can provide more reliable quantitative information about TCMs or more specific information correlated with quality characters. Two main acquisition modes can be differentiated: MRM and SIM. The first two methods have already been widely used; however, given the complexity of TCMs, some innovative methods have also been proposed.

#### 3.3.1. Multiple reaction monitoring

MRM using a triple-quadrupole LC-MS/MS system, which monitors both the specific precursor ions and the characteristic product ions of each analyte, has become a general method that is widely

utilized for the simultaneous quantitation of multiple low-abundance metabolites. However, some bottlenecks exist in the MRM method, such as the concentration span, polarity range, limited number of transitions handled in a run [75], and a lack of authentic references [76]. To resolve the concentration span of components in *Glycyrrhiza* species (rhizome, Chinese name: Gan-cao), 151 bioactive secondary metabolites in samples from three origins were quantified by seven methods, in which the minor components were determined by UPLC/MRM methods. Through correlation with a DNA barcode analysis, it was found that the hybridization of licorice could remarkably alter the chemical composition of licorice, and that the male parent contributed more to the offspring than the female parent [77]. For a wide polarity range or enantiomers, two different columns are adopted with a successive passing-through mode [75] or a heart-cutting 2D mode [78]. RP LC and HILIC columns were used to definitively determine 21 compounds in *Cistanche salsa* (herba, Chinese name: Roucongrong) with the successive passing-through mode [75,79], and achiral-chiral columns were combined to simultaneously quantify 18 coumarins, including seven pairs of enantiomers in *Peucedanum praeruptorum* Dunn (radix, Chinese name: Qianhu), with the heart-cutting 2D mode [78]. Scheduled MRM or dynamic MRM was used to increase the transitions, which allowed more compounds to be determined with improved sensitivity and a better limit of detection (LOD) and limit of quantitation (LOQ), including 41 components in the Niuhuang Shangqing pill [80], and 221 ginsenosides in ginseng and American ginseng [76]. With the lack of authentic references, novel methods are required to resolve the optimization of MRM parameters and absolute quantification. Quantitative <sup>1</sup>H NMR (q<sup>1</sup>H NMR) spectroscopy combined with chromatographic fractionation was used to generate the pseudo-mixed standard solution, which permits more compounds to be quantified [79]. Using the stepped MS<sup>All</sup> (sMS<sup>All</sup>) technique on a quadrupole time-of-flight (QTOF) instrument, MRM parameters with no standard could be predicted and transferred to a triple-quadrupole MS system [81]. Similarly, the MS2 of IT/MS could be used to produce major parameters for MRM, which offered rapid and direct transition design in a quantitative assay [82].

### 3.3.2. Selective ion monitoring

The development of SIM method is driven by the need to quantify special compounds to provide supporting data for the quality control of TCM [83] or for qualitative analysis and identification studies [84,85]. Compared with the MRM method, SIM tends to give reasonable selectivity, but better applicability, when using compact single-quadrupole MS, such as the ACQUITY QDa MS system. The QDa MS is an ideal tool to perform SIM due to its fast switching between ES<sup>-</sup> and ES<sup>+</sup>, few parameter settings, lower cost, and less space occupation [86]. The proposed monomethod-heterotrait matrix method mainly relies on the SIM method, and will be described later in this review.

### 3.4. Supercritical fluid chromatography

SFC is a traditional technology in which supercritical fluids with low viscosity and high diffusivity, such as supercritical carbon dioxide (sCO<sub>2</sub>), are used as a mobile phase. As the technique has progressed, its repeatability and robustness are now much better, resulting in technology that is more applicable for TCMs, such as ultra-high performance supercritical fluid chromatography (UHPSFC). This technique has several interesting features, including high separation efficiency, high mobile phase velocities and thus reduced analysis times, and eco-friendly characteristics. The application of SFC in TCM has recently been reviewed [87]; here, we mention some special applications.

### 3.4.1. Lipidomics for TCM

Lipidomics, the global analysis of a large number of lipids in a matrix, relies heavily on new developments in separation science and technology. SFC-MS can increase lipid coverage while decreasing the cost and the analysis time, and can therefore provide an alternative to other analytical techniques [88]. The lipidomes extracted from three congeneric *Panax* species (*P. ginseng*, *P. quinquefolius*, and *P. notoginseng*) by methyl tert-butyl ether were comprehensively profiled and compared by UHPSFC/QTOF-MS. The samples were analyzed on a 1.7 μm particles packed Torus 2-picolyamine (2-PIC) column using CH<sub>3</sub>OH (in CO<sub>2</sub>) as a modifier and CH<sub>3</sub>OH/0.2 mmol·L<sup>-1</sup> ammonium acetate as the makeup liquid. Six lipid subclasses were well separated, and better resolution of the polar lipids and the lipid isomers compared with an RP system was achieved. A total of 24 triacylglycerols were unveiled based on the analysis of 60 batches of ginseng samples [89]. This technique was also used to explore the lipid markers of Coix seeds from different geographical origins; in that case, diglycerides were the main quality markers [90].

### 3.4.2. Polar chemical components of TCMs

In addition to lipids, polar chemical components of TCMs have been reported through the effective application of SFC. The hydroxyl group at the C-22 position of furostanol saponins is active and easily reacts with lower alcohols. Ten similar structures of furostanol saponins were able to be well separated in 22 min on a diol column at a temperature of 40 °C with methanol (containing 0.2% NH<sub>3</sub>·H<sub>2</sub>O and 3% H<sub>2</sub>O as a modifier) [91]. SFC has also been effective in separating the spirostanol saponins, which share the same aglycone and vary in their sugar chains, and is very sensitive to the number and the position of the hydroxyl groups in the aglycones. However, the resolution of spirostanol saponins with different aglycones and the same sugar moiety by UHPSFC was not ideal, and could be resolved by UHPLC instead. UHPLC was effective in differentiating the variation in aglycones, and was shown to be influenced by double bonds in the aglycones [92]. Eight phenolic acids in *Xanthium sibiricum* Patr (fructus, Chinese name: Cangerzi) were quantified within 25 min on a BEH column with methanol/acetonitrile (70:30 v/v), 1% trifluoroacetic (TFA) as the modifier, and a flow rate of 0.8 mL·min<sup>-1</sup> [93]. Regarding flavonoids, the five flavonoids (kaempferol, luteolin, querctein, luteoloside, and buddeoside) present in *Chrysanthemum morifolium* Ramat (flos, Chinese name: Jühua) were quantified in 15 min on a Zorbax Rx-SIL column with 0.1% phosphoric acid solution in methanol as the polar mobile phase [94]. For seven pairs of 25R/S-ergostanes from the medicinal mushroom *Antrodia camphorata* (Chinese name: Niuzhangzhi), a Chiralcel OJ-H column (4.6 mm × 250 mm, 5 μm, chiral) and a Princeton 2-ethylpyridine (2-EP) column (4.6 mm × 250 mm, 3 μm, achiral) held different advantages for analysis. The chiral column effectively separated each pair of compounds, while the achiral column was able to effectively separate the individually different pairs, although the resolutions of the 25R/S forms of each epimeric pair were not as good as with the OJ-H column [95]. Three types of triterpenoid saponins (kudinosides, staunatosides, and ginsenosides) were also optimized on SFC-MS [96]. To comprehensively determine the metabolites profiling performance of SFC, 120 highly diverse natural compounds (according to lipophilicity, hydrogen bond capability, acid-base properties, molecular mass, and chemical structure) were screened on a set of 15 rationally chosen stationary phase chemistries. Three stationary phases (diol, not end-capped C<sub>18</sub>, and 2-EP) were found to be suitable for untargeted scouting analysis and method development, since they permitted the suitable elution of 101 out of 120 natural compounds. End-capped T3 C<sub>18</sub> and polar P-PFP were found to provide extended selectivity for specific natural molecule subclasses [97].

### 3.4.3. Preparation of standard compounds

The preparation of high-purity reference standards is key in the quality control of TCMs. However, due to rapid configurational changes that can occur in polar or aqueous solvents or due to thermal instability, some natural compounds cannot be obtained in high purity and could not be used as reference standards. Two pairs of 7-epimeric spiro-oxindole alkaloids—rhynchophylline and isorhynchophylline, and corynoxine and corynoxine B from *Uncaria macrophylla*—were separated on two achiral Torus 1-aminoanthracene (1-AA) and Torus DIOL columns using a water-free mobile phase, respectively [98]. Given the good orthogonality, an offline 2D SFC–RP LC system was used to successfully separate 12 lignans from *Arctium lappa* L. (fructus, Chinese name: Niubangzi) [99].

### 3.5. Data mining of large database sets

High-dimensional large databases can be obtained from the comprehensive untargeted analysis of TCMs using the LC–HRMS or SFC–HRMS methods. It was very difficult to glean data-mining information from these large database sets, even compared with metabolomics, because the chemistry in the TCMs showed significantly more diversity than that in humans. In addition, fewer scientists participate in this field (which includes natural products, food chemistry, and herbal medicine), so fewer specific databases could be obtained. Consequently, many different bioinformatics strategies have been used to try to mine data from the massive datasets [100]. Here, we introduce three aspects of data mining.

**Data matching** is routinely performed as a first step to identify potential compounds by searching chemical libraries using the accurate mass or the molecular formula. However, the library-based identification of plant metabolites is particularly challenging due to the limited number of structures contained within the libraries. Metabolite prediction algorithms could use predefined substructures or “building blocks” in enumeration in order to predict the presence of structural components in metabolites; this would increase the chemical coverage space of the available libraries, thereby enabling more efficient, accurate structure dereplication and prediction. This method is especially suitable for structurally diverse glycoside moieties, such as those present in saponins and in glycosylated flavonoids, which typically consist of an aglycone conjugated with various glycosyl and acyl groups [101]. Using the commercial software UNIFI or the in-house software PlantMAT, ginsenosides modified with different glucose, xylose, rhamnose, and malonyl units with one-step modification and two-step modification were produced in a library. With this method, many more ginsenosides (721 for the modified library and 282 for the traditional library) from the leaves of *P. notoginseng* (Chinese name: Sanqiye) were matched [15]. This method might also be used for lignins and polyphenols (tannins and procyandins) [101].

**Data filtration/classification** is still a challenging task based on the large amount of information contained in the raw LC–HRMS data, including MS1 and the MS/MS or MS<sup>n</sup> data. Many post-acquisition data-processing strategies have been developed to conduct data filtration or classification. For MS1 data, significant and ubiquitous matrix interference makes it more difficult to characterize the minor components. Therefore, an integrated filtering strategy and R script was proposed using a combination of different filtering methods, including nitrogen rule, mass defect, and neutral loss/diagnostic fragment ion filtering. The strategy successfully and rapidly screened 16 methoxylated flavonoids and 55 chlorogenic acid analogs from the raw UPLC–HRMS dataset of folium *Artemisiae argyi* (folium, Chinese name: Aiye) [102].

For MS/MS data, a great deal of structural information is contained in the fragment ion information. Thus, a key ion filtering

strategy was proposed to filter and classify different flavonoids from *Scutellaria baicalensis* (Huangqin), and a total of 132 compounds were identified from Huangqin, 59 of which were reported for the first time [103]. Similarly, triterpenoid saponins in *Clematis chinensis* Osbeck (rhizome, Chinese name: Weilingxian) [104], shikonins and shikonofurans in *Arnebia euchroma* (radix, Chinese name: Zicao) [105], and constituents of *Eucommia ulmoides* Oliv (cortex, Chinese name: Duzhong) [106] and *Tribulus terrestris* (fructus, Chinese name: Jili) [107] were classified by the diagnosed product ions. Another efficient classification method from massive MS/MS datasets is the GNPS (<http://gnps.ucsd.edu>), an open-access knowledge base for community-wide organization and the sharing of raw, processed, or identified tandem mass (MS/MS) spectrometry data [108]. This can be used to create a molecular network using a modified cosine scoring scheme that determines the similarity of two MS/MS spectra and visualizes the classification of all the massive MS/MS data; it would be a valuable tool in TCM chemistry research [109].

For MS<sup>n</sup> data, the mass spectral tree similarity filter (MTSF) technique in Mass Frontier software has been used to discover compounds and obtain sub-structure information. This technique can filter useful HRMS and MS<sup>n</sup> data, and establish linkages between unknown and templated compounds, by calculating the similarity match score. It was successfully used for the exclusion of irrelevant ions in the analysis of Erxian decoction [110].

For the mixed mode, different fragment modes can provide complementary fragment ion information. By combining traditional CID-MS<sup>3</sup> and HCD-MS<sup>2</sup> in a LTQ-Orbitrap, diagnostic product ion (DPI) filtering and NLF were used to classify different types of aglycones and sugars of flavonoid O-glycosides from *C. tinctorius* (Honghua) [22].

**Data mining** with chemometrics has become a widely tool in the quality study of TCMs. Principal components analysis (PCA) [111–116] and (orthogonal) partial least squares discriminant analysis ((O)PLS-DA) [90,117–120] are the most frequently used unsupervised and supervised methods of data mining. Quality markers corresponding to different species [77,121,122], different parts of the plant [18,123], different geographical origins [90,111,119,124], different “pao zhi” techniques (which is the technique of altering the properties of crude medicines by processing using heat and combination with various materials in a kind of alchemical approach to preparation) [112,125,126], different vendors [116,127,128], and different batches [118] could be recognized. These quality markers could then be used to predict the quality of a TCM by artificial neural network (ANN) [123] or SVM [129]. The PCA could also be used to discover novel compounds [110,130]. Twelve total ion chromatograms of *Curcuma longa* (rhizome, Chinese name: Jianghuang) were obtained with 12 different NL/precursor ion (PRE) scanning on a triple-quadrupole mass spectrometer. PCA analysis then prompted those data points with similar NL/PRE patterns to be gathered before their molecular weights were revealed, and those compounds with distinct NL/PRE patterns were easily recognized as outliers that might have novel structures [130].

## 4. Application for TCM quality control

With a deeper understanding of the chemistry of TCMs, it should be possible to identify which quality markers should be used as quality standards for a TCM. To save on the use of reference standards and testing time, two quality strategies are proposed.

### 4.1. The SSDMC method

The SSDMC method only needs a single reference standard, and enables the determination of more than 10 components

simultaneously. Although the method is convenient, the conversion factors are most sensitive to the UV detector and peak measurement parameters. In addition, the concentration of the standard solution used to calculate the conversion should be in a suitable range [7,131]. Thus far, the SSDMC method has been widely adopted in the quality control of TCMs, including seven triglycerides in *Coix lacryma-jobi* L. (semen, Chinese name: Yiyiren) [111], six alkaloids in *Aconitum carmichaelii* (Fuzi) [112], nine ginsenosides in ginseng [132], four flavonoids in *Scutellaria baicalensis* (Huangqin) [113], 17 triterpenes in *Ganoderma* (Chinese name: Lingzhi) [133], 11 components in *Gastrodia elata* tubers (rhizome, Chinese name: Tianma) [124], four tanshinones in tanshinones extract [134], six components in *Lonicera* flowers (flower, Chinese name: Jinyinhua) [135], four compounds in *Polygonum cuspidatum* (rhizome and radix, Chinese name: Huzhang) [131], four alkaloids in *Mahoniae Caulis* (ramulus, Chinese name: Gonglaomu) [136], three phenolic compounds in *Rhodiola crenulata* H. Ohba (rhizome and radix, Chinese name: Hongjingtian), and 13 components in *Euphorbia kansui* (radix, Chinese name: Gansui) [126]. The wavelength program [126], evaporative light scattering detector (ELSD) [111], and MS detectors have been used in the SSDMC method [137]. Regarding the MS detector, however, more influence factors should be considered before using it as a standard method. In addition to SSDMC, quantitative analysis by standardized reference extract (QASRE) and quantitative nuclear magnetic resonance (qNMR) spectroscopy have unique advantages in saving reference standards. Each method has its drawbacks and should be applied in specific practices [136].

#### 4.2. The monomethod-heterotrait matrix method

CPMs, which are composed of several to tens of TCMs, are the main usage forms of TCMs. On the one hand, CPMs greatly enrich the application of TCMs and promote personalized medications; on the other hand, they form extremely complex matrices, which therefore hamper quality control. Considering the great number of CPMs and the difficulties in their quality control, it will be time-consuming and rather difficult to construct qualitative and quantitative methods for each CPM. Therefore, a monomethod-heterotrait matrix method has been proposed to facilitate the identification and quantitative assay of the same TCM in different CPMs.

##### 4.2.1. Identification

The main chemical identification method for the quality control of CPMs is thin layer chromatography (TLC) or high-performance thin layer chromatography (HPTLC). The low sensitivity and poor peak capacity of TLC or HPTLC indicates that different methods should be developed for the same TCM in different CPMs. Therefore, these situations would lead to complicated sample preparation procedures and long-running tests, or even to a situation in which no identification method could be performed.

*C. tinctorius* (Honghua) is commonly used in a number of CPMs, but few CPMs involving *C. tinctorius* require its identity to be assured, due to its low content in CPMs. Thus, through the chemical profiling analysis of 20 batches of *C. tinctorius* by UHPLC/QTOF-MS, followed by a thermostable test, six quinonechalcone C-glycosides were chosen as chemotaxonomical markers. Next, a sensitive and highly specific SIM approach based on these six markers was developed to identify *C. tinctorius* from 28 different CPMs. Surprisingly, in 10 batches of the samples, detection of all six markers was not possible, and for two of them, hardly any of the six markers were detected [84]. In a similar manner, for the identification of all three TCMs in Shuxiong tablets, including *P. notoginseng* (Sanqi), *Rhizoma Chuanxiong* (rhizome, Chinese name: Chuanxiong), and *C. tinctorius* (Honghua), three tedious TLC tests

were used in ChP 2015. Using a QDa MS detector, 11 markers covering the three TCMs were selected to evaluate 12 batches of Shuxuetong tablets. In only a single run, all three TCMs could be identified, showing that the QDa MS technique had the same identification capacity as QTOF-MS [86].

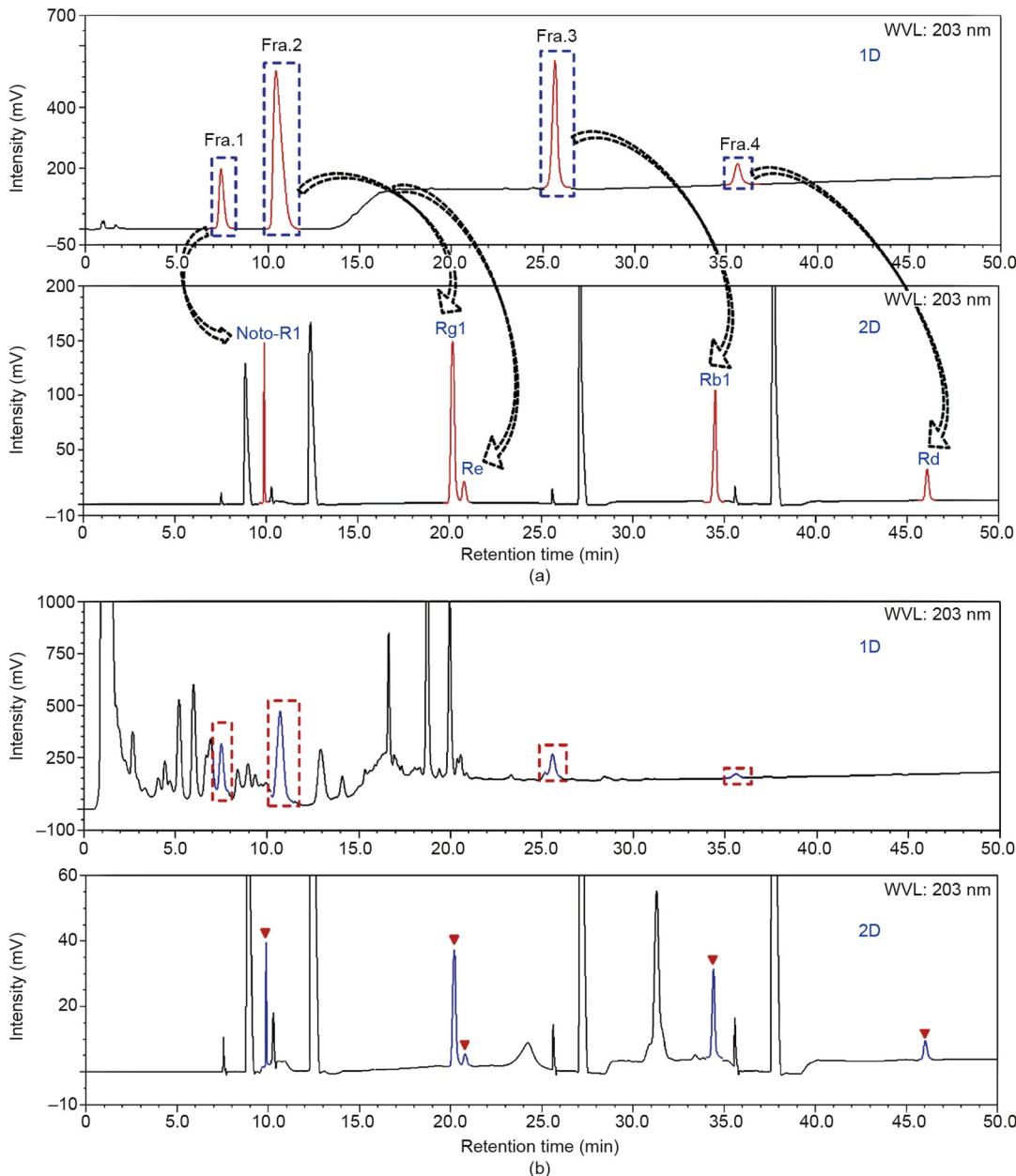
##### 4.2.2. Quantitative assay

Quantitative assay of the same TCM in different CPMs involves complicated sample-preparation procedures and case-dependent chromatographic parameters. Therefore, it is important to develop a universal method in order to achieve the simultaneous quality evaluation of different CPMs involving the same plant.

*P. notoginseng* (Sanqi) is a common TCM used in many CPMs. Notoginsenoside R1 and the ginsenosides Rg1, Re, Rb1, and Rd are the main active components, and are thus considered to be the marker components. ChP 2015 has compiled more than 80 CPMs involving *P. notoginseng*, among which 11 CPMs included *P. notoginseng* as the monarch drug. For some of these CPMs, no quality control, or quality control with limited markers and case-dependent chromatographic conditions, was observed. A heart-cutting 2D LC system was constructed to provide simultaneous assays of five marker saponins in eight different CPMs. Due to the high peak capacity of 2D LC, the sample preparation method was also simplified and unified. The approach was validated in terms of specificity, linearity, precision, stability, and accuracy, and displayed the advantages of high specificity, desirable resolution, and high analytical efficiency. For those CPMs with the same quantitative marker, the unifying method greatly facilitated quality control (Fig. 4) [12].

## 5. Perspectives

This review systematically summarizes the latest technical and methodological progress in the untargeted and targeted analytical chemistry of TCMs, and describes two quality control strategies for the development of TCM quality standards. With the progress in hyphenated analytical methodologies, such as LC–HRMS, LC–solid-phase extraction (SPE)–NMR, and SFC–HRMS, more minor components in TCMs can be recognized easily and rapidly. For untargeted analysis, offline 2D LC–HRMS is the most powerful method to uncover multiple components in TCMs, especially in combination with IM. More improvements in automatic and standard operations, and in computer-assisted data mining, would increase the repeatability and decrease the data analysis time of this method. It should be pointed out that most of the untargeted studies focused on only a few types of secondary metabolites in TCMs, such as saponins, flavonoids, alkaloids, phenolic acids, and terpenes, which are frequently assumed to be associated with biological activity. However, more attention should be given to the other abundant components in TCMs, such as the tannins, polysaccharides, lipids, and proteins, which might also contribute to the pharmacological activity and therefore to the quality. For comprehensive online 2D LC–HRMS, improvement in the analysis time of the second dimension column would significantly increase the peak capacity. Regarding the heart-cutting 2D LC method, better integration of the interface might make this method more practical for the quality control of CPMs. The IM technique gives the constituents an additional parameter in LC–MS analysis, which is extremely useful for identification. Enhancing the separation of IM would make it much more powerful in the chemical analysis of TCMs. Use of the set of DDA modes in LC–HRMS analysis is more difficult when maximizing exposure for the true components. However, only a specific type of compound could yield fragment ion information for DDA. DIA is an untargeted mode to generate MS/MS fragments; it requires computational methods to resolve



**Fig. 4.** Illustration of the monomethod-heterotrait matrix method with a heart-cutting 2D HPLC-UV system. (a) 1D and 2D HPLC-UV chromatograms (203 nm) of five reference standards: notoginsenoside R1 (noto-R1), ginsenosides Rg1, Re, Rb1, and Rd; (b) a Yaobitong capsules sample (a Chinese patent drug with eight herbal medicines, one of which is *P. notoginseng*), showing the four-time transfers between the 1D fractions and 2D separation. Dashed boxes show the heart-cutting time span of four transferred 1D fractions. Fra.: fraction; WVL: wavelength. Reproduced from Ref. [12] with permission of Elsevier B.V., © 2015.

the fragment ion information. More innovative methods are necessary in the DIA field. For target analysis, practicability and coverage are the two primary aspects. Wider coverage of MRM with better validation would give more reliable information for correlation with the quality information of TCMs. The practicability of SIM using a compact and robust mass spectrometer might result in a revolution in the mode of TCM quality standards within the industry. With improvements in the repeatability and robustness of SFC-MS, more chemical analyses could use this technique, once more special columns are designed. Computer languages such as R, python, or MATLAB are very useful in understanding and processing the large database sets obtained from LC-HRMS. GNPS is a very useful platform for the classification of MS/MS data and for data matching. Artificial intelligence (AI) must become the most important tool for data mining in the near future. With a deep understanding of the chemistry of TCMs, all quality-related chem-

ical information could be holographically contained in the substance database.

Test cost and test time are two important factors in evaluating the practical utility of a TCM quality standard. More applications and standard specification should be performed using the SSDMC method and the monomethod-heterotrait matrix method. Use of the concept of moving from comprehensive research to a simplified standard would lead to better TCM quality standards and would more easily translate TCMs into product.

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### Compliance with ethics guidelines

Jin-Jun Hou, Jian-Qing Zhang, Chang-Liang Yao, Rudolf Bauer, Ikhlas A. Khan, Wan-Ying Wu, and De-an Guo declare that they have no conflict of interest or financial conflicts to disclose.

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