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Recent Development in Liquid Chromatography/Mass Spectrometry and Allied Topics for Traditional Chinese Medicine Research

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[ABSTRACT] Traditional Chinese medicines (TCM) have been widely used for the prevention and treatment of various diseases for thousands of years in China, due to their less side-effect, proven efficacy and low cost. But the complexity of the components is still a bottleneck for its modernization and globalization. This paper reviews recent developments in liquid chromatography/mass spectrometry (including quadruple, ion traps, time-of-flight mass analyzers and hybrid instruments) and data processing techniques in the qualitative and quantitative analyses of TCMs. In addition, several hot topics in TCM research such as quality control, fingerprint, multi-component qualification and quantification, pharmacokinetics, and metabolomics are also discussed. Finally, future directions of LC/MS for research on complex systems, such as multiple ionization mass spectrometry strategy, multiple dimensions LC and nanospray LC are also discussed concisely.

[KEY WORDS] Traditional Chinese medicine; Liquid chromatography/mass spectrometry

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1 Introduction

TCM is gaining increasing popularity as an alternative approach in the development of pharmaceuticals and therapeutic applications due to their reliable therapeutic efficacy. Natural medicines provide valuable resources to meet the requirements for global health care [1]. Therefore, safety and efficacy need to be proven in a manner comparable to conventional drugs, and modernization of TCMs requires the use of novel methods and high-tech instruments. Applications of liquid chromatography/mass spectrometry (LC/MS) in the TCM research have been growing rapidly in recent years [2-5]. Owing to rapid technical advances and increasing availability of instrumentation, of the many techniques for the study of natural medicine, LC/MS is becoming one of the most essential one. In this review, no attempt is made to exhaustively review the literature on traditional Chinese medicine (TCM) research by LC/MS, the emphasis will be providing an overview of the most recent advances in the LC/MS technologies for the TCM and natural medicine studies, and highlighting hot topics with several examples.

2 MS Technologies for TCM Research

A variety of techniques have been developed to measure the mass-to-charge ratio (*m/z*) of gas-phase ions. The most common methods include separation in time based on ion velocity and flight distance (time-of-flight); transmission through an electro-dynamic field (quadrupole mass filter); dispersion based on ion momentum or kinetic energy (magnetic and electric sector instruments); and periodic motion in a magnetic or electro-dynamic field (ion traps) [6]. Recently, a significant shift from using single mass analyzer towards employing hybrid mass analyzers was noticed. Hybrid MS/MS instruments use various combinations of different analyzers to obtain desirable performances, such as higher resolving power, better accuracy, sensitivity, and faster analysis, *etc*. The development of hybrid instruments was also motivated by the demand for fast MS^n spectral acquisition required by the time scale of a fast chromatographic separation [7-10].

2.1 *Quadrupole mass spectrometry*

Due to low cost, simple operation and relatively smaller size, low resolution mass spectrometers have been commonly

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used in research to measure the components of TCMs.

2.1.1 *Quadrupole mass spectrometer (QMS)*

Quadrupole mass spectrometers have been used widely for electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) as the interface for LC/MS. Liang *et al* utilized LC/ESI-MS in the positive ion mode to identify six predominant C21 steroidal saponins from *Radix Cynanchi Atrati* [11]. Similarly, ultra-performance liquid chromatography (UPLC)/ESI-MS in the positive ion mode was also applied to determine the saponins in the adventitious roots of *Panax notoginseng* [12]. Chang *et al* established an advanced LC/ ESI-MS method to simultaneously quantify eight active compounds in *Sheng-Mai San* [13]. The 10 major active components in *Ginkgo biloba* extract were quantified with negative LC/ESI-MS in selected ion monitoring (SIM) mode, which provided good accuracy and reproducibility [14]. Chen *et al* utilized LC/MS in the negative ionization mode with SIM data acquisition to quantify the major flavonoids, iridoid glucosides and saponins in *Flos Lonicerae* [15].

2.1.2 *Triple-stage quadrupole MS (TQMS)*

The detection limit for SIM based on QMS is usually determined by how well one could discriminate between target and other components and/or background ions produced by the sample. If one needs MS/MS capabilities for structure determination or for improving selectivity and sensitivity of the target components in the selected reaction monitoring (SRM) mode, TQMS should be used for these purposes.

TQMS techniques play an important role in detection, identification and quantification of TCM components. These instruments have greater capability in discriminating against the co-eluting species and chemical background, resulting in real gains in selectivity and sensitivity for quantitative analysis. The TQMS presents the advantage of versatility in screening because it can operate in full-scan, neutral loss (NL), precursor ion (PI), SRM or multiple reaction monitoring (MRM) and product ion scan modes, which was widely used in the determination of multi-components in TCMs in recent years. For example, LC equipped with TQMS was applied for simultaneous quantification of four alkaloids of *Lindera aggregate* by Han *et al* [16]. LC/TQMS combined with accelerated solvent extraction was adopted to identify and quantify the major alkaloids in the extracts of *Coptis chinensis* Franch in full scan and MRM mode, respectively [17]. Similarly, Li *et al* developed an UPLC/ TQMS method to characterize and quantify 14 synthentic anti-diabetic drugs in adulterated Chinese proprietary medicines and dietary supplements [18]. A high-performance liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI- MS/MS) method was used for preliminary identification and screening of active coumarins in *Radix Angelicae Dahuricae* [19]. Sun *et al* utilized an HPLC-MS/MS in MRM mode to determine the terpenoid lactones in *Ginkgo biloba* [20]. Yang *et al* developed an HPLC/TQMS method in the MRM mode to rapidly quantify the iridoid glycosides in the formulated Chinese medicine *Longdan Xiegan Decoction* [21].

2.2 *Ion traps mass spectrometry*

2.2.1 *Quadraploe Ion Traps (3-D and 2-D ion trap)*

Quadruple ion traps, both 3-D (simply called ion trap, IT) and 2-D (also called linear ion trap, LIT) ion traps are normally operated as the scanning mass spectrometers. Factors affecting the detection limit are the efficiency of the ion injection and trapping, and the duty cycle. The number of ions that can be trapped places a limit on the dynamic range of the trap, that is, the range between the smallest number of ions which can be detected and the largest number of ions that can be stored in the trap. The space charge effects can cause frequency shifts as the number of trapped ions increases and affect the accuracy and precision of mass measurement. The advantage of the ion traps is the possibility for MS^n scan. Collision induced dissociation (CID) is accomplished in the ion traps by applying an excitation waveform which accelerates and then decelerates the ions in the presence of a collision gas. The slow and multiple collisions of the ions lead to highly efficient, low energy CID. The product ion mass spectra recorded with an ion trap tend to be dominated by a few abundant peaks, and multistage mass spectrometry (MS^n) is necessary to obtain detailed structural information. An HPLC–DAD–ESI-MS^n method was established for simultaneous determination of the major chemical constituents in "*QI-SHEN-YI-QI*" dropping pill, a TCM widely used for treating cardiovascular diseases [22]. In this method, an ion trap mass analyzer was applied for MS and MS^n analyses. An HPLC-DAD-MS method using a LCQ ion trap instrument was used for quantitation and qualitation of bioactive phenols in *Dendrobium aurantiacum var. denneanum* [23]. Ye *et al* utilized HPLC-APCI-IT techniques to analyze the bufadienolides in the Chinese drug *Chan Su* [24]. IT mass spectrometry was also used by Han *et al* for the qualitative and quantitative analysis of *Xanthium* fruit, a commonly used TCM [25]. An HPLC/ITMS instrument was used to identify 10 hydrophilic and 9 lipophilic components from Danshen root (*Salviamiltiorrhiza Radix et Rhizoma*) [26]. Similarly, Guo *et al* characterized the phenolic compounds in the fruits of *Forsythia suspense* based on an HPLC/ITMS method [27].

2.2.2 *Triple quadrupole-linear Ion Trap mass Spectrometer (QTrap)*

QTrap combines the advantages of a triple-quadrupole mass spectrometer with those of an ion trap mass spectrometer in the same platform [28-29]. Importantly, this hybrid mass spectrometer has MS/MS scan functions of both traditional triple-quadrupole instruments and linear ion trap instruments, such as NL, PI, MRM scan, full-scan MS-dependent MS/MS analysis and the acquisition of MS^3 spectra [30-31]. Thus, it has been widely used in TCM studies. Jin *et al* developed a QTrap method to monitor the precursor and product ions of the analytes in MRM mode to simultaneously qualify and

quantify 19 diterpenoids in *Isodon amethystoides* [32]. Wang *et al* developed a LC/QTrap method employing both positive and negative electrospray ionizations in the SIM and MRM modes for simultaneous analysis of nine compounds in the raw herbs and products of *Si-Wu-Tang* [33]. LC/QTrap in multiple ion monitoring-information dependent acquisition-enhanced product ion mode (MIM-IDA-EPI) and precursor ion scan-information dependent acquisition-enhanced product ion mode (PI-IDA- EPI) were employed for characterization of coumarins in *Radix Glehniae* [34]. With a hybrid triple quadrupole/linear ion trap mass spectrometer, multiple endogenous plant hormones in leaf tissue of *Oliseed Rape* were quantified in the MRM mode and identified in the IDA mode with high sensitivity and selectivity [35].

2.3 *Time-of-flight mass analyzers (TOF, Q-TOF and IT-TOF)*

Mass accuracy, which is defined as MA = $10^6 \times (m/z_{exp} - m/z_{theor})/(m/z_{theor})$, where m/z_{exp} is the measured value and m/z_{theor} is the theoretical value, is extremely important for application of high resolution mass spectrometry to the analysis of TCMs. With external calibration, values less than 5 ppm were provided by modern high mass accuracy instruments in routine use. The determination of accurate masses is crucial not only for identifying the right elemental formula from different predicted results, but also for decreasing the number of possible formulae, which is vital for the characterization of the complex composition of TCMs. In addition, both MS and MS^n modes for molecular adducts and fragment ions, have been provided to obtain the most probable elemental formula.

2.3.1 *TOF*

For TOF detectors, from its flight time through a field free drift region (flight tube) of a specified length under vacuum, the *m/z* of a ion could be calculated. The ions with various masses and similar energies, are separated according to velocity when passing down the flight tube, with the ions of lower masses reaching the detector earlier. Two types of data acquisition systems, time-to-digital converter (TDC) and analogue-to-digital-converters (ADC), are used in TOF mass spectrometers. The dynamic range of the TDC is limited by the counting dead time-the period after each ion event when the TDC itself is unable to register another count. In each TOF pulse, only one ion can be recorded during a dead time so that if two ions with the same *m/z* value arrive at the detector, only the first one is recorded. The result is that intense mass peaks become distorted by depletion of the top and right side of the peak, so that the peak intensity is suppressed and the centroid shifted to the left.

Due to high full spectrum detection sensitivity, mass accuracy and data acquisition rate, TOF mass spectrometers are widely used in the field of TCM analysis [36-38]. Shi *et al* established a reliable method using combined HPLC/TOFMS and HPLC/ITMS to investigate miscellaneous components in *Ixeris sonchifolia* (Bunge) Hance (a folk medicine widely used in China for its anti-inflammatory and haemostatic effects), including six sesquiterpene lactones, six phenolic acids and seven flavonoids [39]. Using a rapid-resolution liquid chromatography with time-of-flight mass spectrometry (RRLC/TOFMS) method, Zheng *et al* investigated 19 oleanane-type triterpene saponins (OTS), the major active ingredients in *Glycyrrhiza uralensis*, to differentiate the subclasses of OTS [40]. To investigate the qualitative profiling of multi-parametric metabolic changes of raw *Panax notoginseng* during the steaming process, a UHPLC/TOFMS method has been established by Toh *et al* [41]. Furthermore, it was also applied to the quality control of *Panax notoginseng* products.

TOF has also been utilized in the characterization and quantification of TCMs in many cases [42-43]. Liu *et al* established a comprehensive method involving combined HPLC-DAD-ELSD and HPLC/TOFMS which could simultaneously quantify the 12 bioactive components in Chinese medicinal prescription *Buyang Huanwu decoction* [44]. Wu *et al* applied an ESI-TOFMS method to support the rapid recognition and structure elucidation of 20 natural iridoid glucosides and phenylpropanoid glycosides in *Radix Scrophulariae* [45]. By combination of HPLC/DAD, HPLC/TOFMS and HPLC/ITMS, two furocoumarins (imperatorin and isoimperatorin) in *Angelica dahurica* extract were identified unambiguously by comparing their relative retention times, characteristic ultraviolet information and accurate mass measurement. A formula database of known furocoumarins in *Angelica dahurica* was established, from which other 21 furocoumarins were identified effectively based on accurate masses of the extracted masses and the formulae acquired by HPLC/TOFMS. To distinguish the isomers, multi-stage mass spectrometry (MS^n, ion trap mass spectrometry) analysis was also performed [46].

Identification of 64 bioactive compounds including flavonoids, triterpene saponins, and monoterpene glycosides in the Chinese medicine formulation PHY906 which is prepared from four medicinal herbs for adjuvant cancer was demonstrated using LC/ESI-TOFMS by Ye *et al* [47]. Recently, Wang *et al* described a methodology based on HPLC/TOFMS to detect 30 chemical constituents in *Zhimu-Baihe* herb-pair, including 24 saponins, three xanthones, one anthraquinone and two alkaloids [48].

2.3.2 *Q-TOF*

Via in-source CID, some TOF analyzers produce structure information, whereas, Q-TOF is advanced to gain more detailed information for the detected compounds. Q-TOF mass spectrometer can be described in the simplest way as a triple quadrupole with the last quadrupole section replaced by a TOF analyzer. For MS measurements, the mass filter Q1 is operated in the RF-only mode so that it serves merely as a transmission element, while the TOF analyzer is used to record spectra. The resulting spectra benefit from high resolution and mass accuracy of the TOF instruments, and also from their ability to record all ions in parallel, without scan-

ning [49].

In the MS/MS mode, the precursor ions selected in the first quadrupole undergo fragmentation through CID in the collision cell. After that, the product ions are detected in the TOF detector [50]. By discriminating between interference and mass peaks of similar nominal masses but various exact masses, Q-TOF analyzers could be used in analysis with relatively high mass resolution and accurate mass measurements, providing a certain degree of selectivity. Hence, it is very suitable for the identification and quantification of the complex systems of TCM [51-53].

Liu *et al* used UPLC/ESI-Q-TOFMS to investigate the fragmentation behavior and mass spectra profiles of the bufadienolide standards. They also characterized new stereoisomers of the known bufadienolides by comparing fragment abundance profiles. Based on the fragmentation rules of the bufadienolide standards, 20 possible novel compounds containing 8 stereoisomers, and 19 known ufadienolides were identified from complex toad skin extracts, which demonstrated that UPLC/Q-TOFMS was a powerful tool to characterize the low-abundance bufadienolides in complex samples [54]. To distinguish different subclasses of *Fritillaria* alkaloids (FAs), the anti-tussive and expectorant herbs widely used in TCM, Zhou *et al* used LC/ESI-QTOF-MS/MS to screen and identify 41 major FAs including 29 cevanine type FAs, one jervine type FAs, six veratramine type FAs and five secosolanidine type FAs [55]. In a successful application of UPLC/Q-TOF for the investigation of TCM, this system has been proven to have great potential for the study of natural medicine [56-58].

MS^E is a new method for LC/Q-TOF data acquisition. MS^E uses an intelligent approach where parallel alternating scans are acquired at either low collision energy in the collision cell to obtain precursor ion information, or high collision energy to obtain full-scan accurate mass product ions, precursor ion and neutral loss information. All data are obtained from a single analytical run. The new UPLC/MS^E approach promises to provide excellent chromatographic and MS efficiencies for the task of structure elucidation in complex mixture analysis. This technique maximized the data collection efficiency of the instrument with no requirement to pre-select an analyte *m/z* value in Q1 for MS/MS fragment ion generation. The use of this alternating collision energy approach maximizes the duty cycle of the instrument ensuring that MS and MS/MS data are obtained for the entire peak in the chromatogram. This is of particular importance when a large number of highly complex samples are analyzed in a short period. Unlike traditional MS/MS, the first quadrupole is not used for mass filtering and thus the MS data of both precursor and product ions are generated under accurate mass conditions. The relevant precursor and product ions are easily linked using either retention times, mass defect or a combination of both. The resulting accurate masses for the precursor and product ions generated from the experiments can then be used to confirm the identity of components in the sample. Another useful feature that derives from the MS^E methodology is that as both precursor and fragment ions are formed in one analytical run, it is possible to screen various chemical classes of components. This is achieved by employing an ion cluster search function post-analysis, giving a pseudo-neutral loss capability for all analytes and eliminating the need for several replicate runs employing various neutral loss criteria [59]. Yan *et al* reported an improved method for UPLC/Q-TOFMS employing Metabolynx XS with mass defect filter (MDF), a post-acquisition data processing software, for global detection of aconitum alkaloids in *Yin Chen Si Ni Tang*, a traditional Chinese medical formula and detected more aconitum alkaloids [60]. Zhu *et al* reported a method based on the retention times, the mass spectrometric fragmentation patterns, and MS and MS/MS data, a total of 31 saponins with 5 aglycone skeletons including 14 new trace saponins were identified or tentatively elucidated in the crude extracts of *D. zingiberensis* [61].

2.3.3 *IT-TOF*

LCMS-IT-TOF consists of a hybrid quadrupole ion trap and TOF mass spectrometer. This instrument successfully combines the MS^n capability of the ion-trap with high-resolution and high-accuracy mass measurement capability of the TOF, which is vital for structure identification of the complex systems, such as TCM, to acquire a great mount of valuable information. Furthermore, without internal calibration, the LCMS-IT-TOF has been proven to provide excellent and stable mass accuracy. And according to the original results obtained by existing customer installations of the LCMS-IT-TOF, the mass accuracy is extraordinary stable over long periods of time. The stability is partly owing to the thermal control of the flight tube, RF and high voltage powers.

Deevanhxay *et al* published a method for simultaneous analysis of quaternary alkaloids, 8-oxoprotoberberine alkaloids, and a steroid in *Coscinium fenestratum* by using LC/IT-TOFMS. A total of 32 compounds including two benzylisoquinolines, three aporphines, 12 quaternary protoberberines, 10 8-oxoprotoberberines, three tetrahydroprotoberberines, and one steroid were characterized [62]. Recently, LC-IT-TOFMS has been successfully utilized for global identification of the target and non-target compounds in TCM, such as *Shengmai* [63] and *Mai-Luo-Ning* injection[64], *Radix Salvia miltiorrhizae* [65], etc. Ten major pregnane glycosides including one novel compound auriculoside IV from the roots of *Cynanchum auriculatum Royle ex* Wight have been firstly detected and identified by a HPLC/IT-TOFMS method [66]. Wang *et al* established a method based on ESI-IT-TOFMS to investigate the fragmentation pathways for rutaecarpine (a major quinazolinocarboline alkaloid isolated from the well-known Chinese herbal drugs *Wu-Chu-Yu* and *Shih-Hu*, the dried, unripe fruits of *Rutaceous* plants, such as *Evodia rutaecarpa* and *Evodia officinalis*) and its derivatives [67].

2.4 *Orbitrap and LTQ-Orbitrap*

In Orbitrap mass spectrometer, the ions are constrained by a combination of electrostatic and centrifugal forces between a spindle-shaped central electrode and two bell-shaped outer electrodes. This instrument is characterized by the use of an Orbitrap mass analyzer preceded by an external injection device based on trapping ions in RF-only gas-filled curved quadrupole (the C-trap). Orbitrap mass analyzer could be utilized in a wide range of applications, from routine compound identification to the analysis of trace-level components in complex mixtures [68]. Compared with many other mass spectrometers, the Orbitrap operates with a higher mass resolution and accuracy over a wider dynamic range [69]. Normally, internal calibrated mass accuracy less than 1 ppm can be achieved. However, the primary disadvantage of the Orbitrap detector is the relatively slow data acquisition. Furthermore, resolving power is directly proportional to the number of detected oscillations while sensitivity is proportional to the square root of this number. For a commercial Orbitrap analyzer, nominal resolving power of 100 000 FWHM (full-width at half-maximum peak height at *m/z* 400) requires a detection time of 1–1.5 s [70-71].

During the past few years, LTQ-Orbitrap, the most recent hybrid trap-trap instrument, has been developed. LTQ-Orbitrap mass spectrometer couples high resolution mass analyzer to a linear ion trap, in parallel, combining accurate mass measurements with the high trapping capacity and MS^n scan function of the linear ion trap. Hence, the acquisition of MS/MS spectra in the linear ion trap instead of the Orbitrap is preferred, and collection of these data in the Orbitrap performing at a lower mass resolution of 7 500 with a scan time of 0.1 s could be applied [72].

The LTQ Orbitrap Velos™ and Exactive™ represent the new development of the Orbitrap techniques. The Orbitrap is used as an accurate mass detector for a linear trap mass analyzer. Even though this instrument is the derivative of the previous described LTQ-Orbitrap hybrid mass spectrometer [73-75], its performance has been improved by many significant modifications. A standard linear ion trap has been replaced by an integrated dual-pressure linear trap, which makes the scan rate faster. Furthermore, there is also a stacked-ring ion guide on atmospheric-to-vacuum interface for increasing transmitted ion currents [76]. The improved efficiency and speed of fragmentation by modifications to the design of the C-trap and the HCD (higher-energy collision induced dissociation) has been reported [77]. With the improvement of this technique, LTQ-Orbitrap has been widely used in the identification of TCMs. The Q Exactive benchtop LC-MS/MS is the next step in that revolution, combining high-performance quadrupole precursor selection with high-resolution, accurate-mass Orbitrap detection for faster scans and polarity switching in both MS and MS/MS, and just announced at 59th ASMS Conference.

To demonstrate the chemical profiles related to the antioxidant activity of *Chaihu-Shu-Gan-San* (CSGS), a TCM formula containing seven herbal medicines, which has been used in treatment of gastritis, peptic ulcer, irritable bowel syndrome and depression clinically. A total of 33 chemical constituents in CSGS were investigated by a method based on LC/LTQ-Orbitrap, which established by Su *et al* [78].

2.5 *FTICR and LIT-FTICR*

The typical features of FTICR mass spectrometry are as follows: ultra high mass resolving power (routine mass resolving power > 200 000), accurate mass measurement, wide mass range, simultaneous detection of all ions, ion re-measurement, ion storage, high resolution ion isolation, and multistage tandem mass spectrometry (MS^n). However, the instrument has to be operated at very high vacuum (10^{-8} Pa) and the added complexity of a cryogenically cooled magnet [79].

In a review, Reemtsma pointed out that ESI combined with FTICR-MS has great potential for the analysis of fulvic and humic acids and of natural organic matter (NOM) at the molecular level. In addition, he also showed many methodical questions in the analytical process before and after the FTICRMS measurement and the major tasks following the generation of data by FTICRMS, which could greatly enhance the benefit of application of FTICRMS in the area of NOM analysis [80]. Huang *et al* demonstrated the application of the ESI-$ITMS^n$ and ESI-FT-$ICRMS^n$ for analyzing a number of dibenzocyclooctadiene lignans and distinguishing the isobaric compounds from the methanolic extracts of *Fructus Schisandrae* [81]. The intrinsic worth of FTICRMS and associated space charge issues were comprehensively reviewed by Zhang *et al* [82], whilst the mass accuracy less than 1 ppm could be achieved using FTICRMS with the implementation of certain external calibration protocols, which has been demonstrated by a recent round-robin study [83]. In addition, ESI-FTICRMS accurate mass measurement has been successfully automated in the pharmaceutical industry to assign the formulae for expected compounds [84].

LIT-FTICR instrument couples a commercial linear quadrupole ion trap (LIT) [85] to an FTICR mass spectrometer [86], featuring both high MS/MS efficiency of LIT and high mass resolving power and accuracy of FTICR instruments. This capability is advantageous to improve the duty cycle and increase dynamic range. Instead of CID, primarily electron capture dissociation (ECD) and infrared multiphoton dissociation (IRMPD), the LIT-FTICR is further advanced by the ability to use ion activation techniques. Nevertheless, the popular application of LIT-FTICRMS instrument is impeded by the drawbacks, such as the cost, maintenance, and space requirement. During the past few years, FTICRMS and LIT-FTICRMS have been successfully applied for identifying metabolites of multi-components in herb medicines [87-89].

3 Post-acquisition Data Mining Approaches for High Resolution Mass Spectrometry

Accurate mass data are produced by a high resolution

mass spectrometer, such as TOF, Orbitrap, or FTICRMS instruments. These instruments have demonstrated a great value for rapid confirmation of the molecular formulae of analytes or for structure elucidation of unknown compounds owing to their high sensitivity and/or MS^n capability. However, unlike triple quadrupole instruments, these hybrid high-resolution mass spectrometers often do not have true NL, PI, and MRM scanning capabilities, and analysis of complex components in TCM, especially at low levels, is often a great challenge because the ions of these components are often masked by background noises. Recently, several new methodologies using high resolution mass spectrometry and post-acquisition data mining techniques have been developed for TCM research.

3.1 *Narrow window Extracted Ion Chromatogram (nwEIC)*

If high mass accuracy can be maintained in the applications throughout the LC/MS experiments, it is possible to extract ion chromatograms (EIC) with a sufficiently high degree of accuracy, so that the overlapping isobaric signals resulting from co-elutes and matrix can be readily separated to find the analyte peak and increase the signal to noise ratio for compound identification and/or quantitation. Such applications typically require at least ± 10 mDa window from the target mass or ± 10 ppm mass measurement accuracy.

Using LC/TOFMS and narrow mass window extraction, two compounds were screened out from the complex matrix of *Radix Scrophulariae* , and identified as cistanoside D and 1-*O*-dihydroxyphenethylol-3-*O*-(demethyl-rhamnopyranosyl) -4-*O*-(feruloyl)-*β*-D-glucopyranoside [90]. Zhang *et al* established a formula database of known furocoumarins in *Angelica dahurica* by the nwEIC, based on the accurate masses and formulae produced by TOFMS, 23 furocoumarins were identified effectively [91].

3.2 *Isotope Pattern Filter (IPF)*

Normally, high-resolution mass spectra contain a second informational dimension beyond the accurate mass and peak intensity (relative ion abundance), the spectral isotope distribution resulting from natural isotope abundance [92]. With introduction of high resolution mass spectrometers, such as Q-TOF, IT-TOF, LTQ-Orbitrap and FTICR, starting from computation of the elemental composition of an unknown compound using accurate mass with errors < 5 $\times 10^{-6}$, many chemically possible formulae can be obtained in higher mass regions. In automatic routines, an additional orthogonal filter therefore needs to be applied in order to reduce the number of potential elemental compositions. Ipsen *et al* [93] develop a method to construct the confidence regions for the isotopic abundance patterns based on the fundamental distribution of the ion arrivals, and moreover, develop a method to make use of the information pooled together from the measurements obtained across an entire chromatographic peak, as well as from any adducts, dimers, and fragments observed in the mass spectra. This greatly increases the statistical power, thus enabling the analyst to rule out a potentially much larger number of candidate formulae while explicitly guarding against false positives. Kind and Fiehn [94] demonstrated that high mass accuracy (< 1 $\times$ 10^{-6}) alone is not enough to exclude enough candidates with complex elemental compositions (C, H, N, S, O, P, and potentially F, Cl, Br and Si). The use of isotopic abundance patterns as a single further constraint removes > 95% of false candidates. This orthogonal filter can condense several thousands candidates down to only a small number of molecular formulae.

3.3 *Mass Defect Filter (MDF)*

The creation of the MDF algorithm was the realization that the mass defects (the non-integral portion of a *m/z* value, MD) of phase I (hydroxylation, dehydrogenation, demethylation, *etc*) and phase II metabolite (glucuronidation, sulfation, *etc*) ions typically fall within 50 mDa relative to that of the parent drug [95]. Based on MD and nominal mass of the parent drug, a MD window (e.g. ± 50 mDa) was set to find the signal of ions whose decimal portion is very similar to that of the parent drug, and to remove the signals of ions whose MD exceed the defined window. MDF has been extensively used for *in vitro* and *in vivo* detection of metabolites. This technology combined with other post-acquisition data processing algorithms has also been used to analyze components in complex natural medicines.

Yan *et al* developed a method employing mass defect filter for global detection of aconitum alkaloids in *Yin Chen Si Ni Tang*, a traditional Chinese medicinal formula. The full-scan LC–MS/MS data sets were acquired using UPLC/Q-TOFMS with the MS^E mode in a single injection. As a result, 62 ions were assigned rapidly to aconitum alkaloids and identified tentatively by comparing the accurate mass and fragments information with that of the authentic standards or by mass spectrometry analysis and retrieving the reference literature [60].

3.4 *Molecular Feature Exaction (MFE)*

The purpose of molecular feature exaction of data processing for LC/MS raw data is to identify all signals for true analyte ions, avoid detection of false positives and provide quantitative information on analyte concentrations. Molecular feature exaction is an essential step of data processing pipeline to study the complex systems, such as TCM, metabolomics, *etc* [96]. The MFE algorithm is a compound-finding technique that locates individual sample components (molecular features), even when chromatograms are complex and the compounds are not well resolved. MFE locates ions that are covariant (rise and fall together in abundance), but the analysis is not exclusively based on chromatographic peak information. The algorithm uses the accuracy of the mass measurements to group related ions—related by charge-state envelope, isotopic distribution, and/or the presence of adducts and dimers. It assigns multiple species (ions) that are related to the same neutral molecule (for example, ions representing multiple charge-states or adducts of the same neutral molecule) to a single compound that is referred

to as a feature. Using this approach, the MFE algorithm can locate multiple compounds within a single chromatographic peak (co-elute). When using mass spectrometry to analyze samples containing unknowns, it is often necessary to derive elemental compositions (molecular formulae) for the unknowns based on the mass spectral data [97]. By using molecular feature extraction, a rapid, sensitive and versatile LC/ESI-TOFMS procedure was developed for comprehensive analysis of the chemical constituents in the Chinese medicine *Venenum Bufonis* (VB, *Chan Su* in Chinese). More than 900 features were detected from VB extracts. Among them, a total of 97 components were identified using the Agilent METLIN accurate mass matching database established according to those reported in the literature. The targeted MS/MS experiments of the 30 major compounds were performed for their quantification and semi-quantification [98].

4 Hot topics of TCM research

4.1 *Quality control*

Due to the complex chemical nature of TCMs, the multifunctional components along with their inherent holistic activities are frequently not clarified, leading to inadequate analysis of the natural medicines by current chromatographic techniques [99]. With frequent usage of TCMs, increased adverse effects that would put the patient's health and safety at risk were reported. Since the effectiveness and quality of TCMs depend on the concentrations of their active ingredients, and significant variations in the active ingredients between herbal samples, causing by numerous factors, such as the climate, cultivation conditions, harvest time, drying, storage, extraction procedure, and deliberate or coincidental adulterations, have been observed, the comprehensive quality control of TCMs is of utmost importance. Although major steps have been taken to improve the quality of the herbal products and sophisticated and advanced techniques have been applied for quality control, assessment and quality control of natural medicines are still a major bottleneck [100].

4.2 *Fingerprint*

The herbal standardizations based on the quantitative analysis of a single marker compound could not reflect the synergistic nature of the herbal medicine, hence, the fingerprinting concept has been proposed for the quality control of the TCMs, characterized by numerous active ingredients and multiple varieties. Since the chemical fingerprints constructed by chromatographic techniques, especially by hyphenated chromatographies, might appropriately represent the "chemical integrities" of the natural medicines, it is strongly recommended to use authentication of the natural medicines to address the issue of quality control for TCMs [101]. The fingerprint of *Eucommia ulmodies Oliver* (*E. ulmodies*) have been constructed by Tong *et al* using HPLC and LC/MS for a comprehensive quality evaluation [102]. Chen *et al* combined UPLC separation with a dual detection system of photodiode array detector (PDA) and ESI-MS/MS to establish of the fingerprints based on distribution of the eight major alkaloids in the extracts of *Coptis chinensis* Franch, which could be a quick and reliable method for the quality control of this herb medicine [103]. To obtain a fingerprint of the furocoumarins from the roots of *Angelica dahurica*, Kang *et al* developed a method based on HPLC-DAD-ESI-MS^n. The results indicated that the method could be used for the quality evaluation of the roots of *Angelica dahurica* [104]. For differentiating the official and non-official *Herba Cistanches* (*Rou Cong Rong* in Chinese), dried succulent stems of *Cistanche deserticola* or *C. tubulosa*, Jiang *et al* elucidated the fingerprint of *C. deserticola* by a HPLC–DAD–MS fingerprinting method, with 18 characteristic peaks investigated [105]. Tong et al published a method based on HPLC and LC/MS to construct the fingerprint of *Eucommia ulmodies Oliver* (*E. ulmodies*), which could be applied to the quality assessment of related herbal medicines [106]. Zhao *et al* established a LC–ESI-MS^n method to investigate the fingerprints of *Psoralea corylifolia* of 10 batches from Sichuan and Henan Provinces, China, and obtained 12 common peaks, which could assess the differences among the herbs grown in various areas of China [107].

To accomplish the quality evaluation of *Ganoderma lucidu*, Chen *et al* identified 19 common peaks in the fingerprint obtained from 29 batches of *G. lucidum* of three different origins in China using HPLC/ESI-MS [108].

Due to high chromatographic resolution, which is necessary for accurate mass measurement, high-resolution MS technologies have also been increasingly utilized in fingerprint analysis of TCMs. Zhou *et al* identified 44 polymethoxylated flavones (PMFs), potential cancer chemopreventive agents, in *Fructus aurantii* (*F. aurantii*) by comparing the mass spectrometric fingerprint (MSFP) of the observed candidates with the diagnostic MSFP of the species, using UPLC/Q-TOFMS/MS [109].

To extract useful information buried in the enormous amounts of data generated by the fingerprint analysis, various chromatography including TLC， HPLC， LC/MS and chemometric methods, such as pattern recognition model, should be combined to assess the data in an objective manner, to further advance the methodology of quality control of TCMs [110]. Ni *et al* constructed characteristic fingerprints of 46 *Eucommia* bark samples originating from different locations and determined some unknown peaks as resinols, geiposidic acid and chlorogenic acid in the samples by combining LC-DAD and LC/MS. Further application of pattern recognition models, such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) for distinguishing differences in the samples demonstrated that the combination of multi-analytical techniques with chemometrics was a powerful tool in the quality control of TCMs [111].

4.3 *Multi-component qualification and quantification*

Qualitative and quantitative analyses could reveal de-

tailed composition of TCMs, accomplish more thorough study on TCMs, and in the mean time, provide systematic and in depth chemical information for more effective quality control of TCMs [112]. It has been well demonstrated that in most cases, TCMs exert their efficacies via synergistic actions of multi-components. Therefore, a multi-component assay might be a rational strategy to elucidate the overall outcomes and to comprehensively control the quality of TCM. Due to its high accuracy for the qualitative and quantitative analyses of TCMs, HPLC coupled with many detectors has been widely utilized in the standardization of TCMs. LC/MS is one of the most essential techniques for qualitative and quantitative analysis of TCM.

Liu *et al* applied LC-DAD-ESI-IT-TOFMS technique for comprehensive and systematic separation and characterization of the bioactive alkaloids in *Sophora flavescens* Ait. A total of 22 constituents were identified on the basis of the extracted ion chromatograms for different $[M + H]^+$ ions of the alkaloids presented. Among these, five constituents were unambiguously identified by comparing their retention times and MS^n spectra with those of the authentic compounds, and 17 other constituents were tentatively identified on the basis of their MS^n fragmentation behaviors and/or molecular weight information in literature [113]. A method based on LC/QTrap technique for the characterization of coumarins in *Radix Glehniae* was developed by Yang *et al.* An extract of *Radix Glehniae* was analyzed by combination of two scan modes, i.e., multiple ion monitoring-information-dependent acquisition-enhanced product ion mode (MIM-IDA-EPI) and precursor scan information-dependent acquisition-enhanced product ion mode (PREC-IDA-EPI). A total of 41 coumarins were identified on the basis of their mass fragmentation patterns. This method allowed the identification of coumarins in *Radix Glehniae* in trace amounts [114].

A LC-MS/MS method for simultaneous analysis of coumarins, ganoderic acids C2, B, A, H, D in *Ganoderma lucidum* and their analoguess was reported [115]. Mass spectrometric detection was achieved by a triple-quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface operating in the negative and positive ionization modes via a within-run polarity switching. Quantitation of five ganoderic acids was performed using the SRM mode, and low levels of ganoderic acids in the fruiting bodies of *Ganoderma sinense* and *G. applanatum* were accurately quantified.

Flos Lonicerae, the flower buds of several medicinal *Lonicera* species, is a commonly used traditional Chinese herbal medicine. A multi-component quality control method using LC-ESI/TOF MS has been developed for simultaneous identification and quantification of 32 bioactive compounds in *F. Lonicerae*. Furthermore, six unknown compounds were tentatively characterized based on accurate mass measurement and CID experiment [116].

4.4 *Pharmacokinetics*

In general, the effective constituents in TCMs can be confirmed by analyzing the compounds absorbed in the blood after oral administration since only the absorbed compounds have the chance to show the effects. Furthermore, many compounds can be transformed to metabolites to show *in vivo* bioactivities under the action of enzymes. The TCMs are complex mixtures containing up to hundreds or even thousands of different constituents, it is extremely difficult to screen and analyze the bioactive components and the active metabolites for pharmacokinetic studies. Fortunately, advances in LC/MS techniques and practical strategies now make the isolation and structural analysis of potent bioactive plant constituents possible. In our lab, Liang *et al* developed a powerful technical platform to rapidly identify and classify metabolites of herbal components based on LC/IT-TOFMS, and completed *in vitro* and *in vivo* metabolic studies for *Schisandra lignans* extract [117]. The major metabolic pathways of *S. lignans* were proposed as demethylation, hydroxylation, and demethylation & hydroxylation after structurally characterizing metabolic pathways of five representative *S. lignans*. In *in vivo* study, 44 metabolites of *schisandra lignans* were detected in rat urine, and were identified and classified rapidly according to the metabolic rules. Park *et al* applied LC-MS/MS in SRM mode to simultaneously determine tanshinone I, dihydrotanshinone I, tanshinone IIA and cryptotanshinone, the active components of *Salvia miltiorrhiza* in rat plasma [118]. An LC-MS/MS method was employed for simultaneous determination of albiflorin and paeoniflorin in rat plasma after oral administration of *Radix Paeoniae Alba* extract and *Tang-Min-Ling-Wan* [119].

4.5 *Metabolomics*

TCM has a long history and been accepted by the academic community and patients as superior and a unique valuable property in China. TCM is facing severe challenges or problems. The largest obstacle suffers from insufficient modern scientific research, not only affecting the status of TCM, but also restricting its development in abroad. Fortunately, as a systemic approach, metabolomics adopts a 'top-down' strategy to reflect the function of organisms from terminal symptoms of metabolic network and understand metabolic changes of a complete system caused by interventions in holistic context [120-121]. This property is in concert with the holistic efficacy of TCM, suggesting that metabolomics has the potential to impact our understanding of the theory behind the evidence-based Chinese medicine [122]. Depending on series of analyses of different sample spectra and combination with chemical pattern recognition methods, metabolomics can be used to identify organisms in pathophysiological state, gene function, drug toxicity and efficacy, and associated biomarkers [123].

Metabolomics allows systematic study of a complex mixture, which can be linked to observations obtained through biological testing systems without isolation of the active principles. Yuliana *et al* [124] discussed the chemical diversity of natural products (NP) compared with synthetic

ones, the obstacles which might limit NP integration with the new emerging drug discovery technologies, the shift in tendency from a reductionist to a holistic approach in NP research and how metabolomics can help to narrow the gap between NP and the demand of modern drug discovery, and help to turn NP and medicinal plants into a unique drug source. This review includes the descriptions of some examples of successful metabolomics applications in several important fields, such as identification and dereplication of metabolites, quality control of phytomedicines, chemical profile and bioactivity pattern of phytomedicines, proof of efficacy and mode of action of herbal medicines, bioavailability and fate of natural compounds assessment, safety and toxicity of herbal medicines assessment *etc*.

Metabolomics, which refers to the untargeted quantitative or semi-quantitative analysis of the metabolome, is a promising tool for biomarker discovery. The use of LC/MS has increased over the recent years, providing complementary results to those obtained through other approaches [125].

From technical point of view, metabolomics is the combination of analytical chemistry, statistics and bioinformatics. The LC/MS based metabolomics methodology include: (i) sample preparation, (ii) acquisition of metabolic fingerprints, (iii) automatic detection of ions (features), (iv) statistical analyses and (v) identification of compounds. However, despite recent technological and conceptual advancements, metabolomics appears to be still in its infancy and each step mentioned above is still a bottleneck [126-127]. Quality control, safety and toxicity assessment and mechanism study are important applications of metabolomics. Song *et al* [128] analyzed large sizes of samples using UHPLC–UV–Q-TOFMS and PCA on the raw material, the in-house prepared aqueous extract of *Radix Salviae Miltiorrhizae* and commercial product, to determine the variation of specific constituents between raw material and the final products as well as the effect of manufacturing process on the overall quality. Ma *et al* used metabolomic techniques for chronic toxicology study of morning glory seed in Wistar rats. The difference in metabolic profiles between the control and the dosed rats was well observed by PCA of the MS spectra. Significant changes of 12 metabolite biomarkers were detected in the rat urine samples. Metabolomics method could discriminate the model rats from the controls in the 2nd, 6th and 10th week respectively, before serious organic damage of kidney was found in the 10th week by histopathology method [129]. A comprehensive metabolomics method, in combination with fingerprint and target analysis, was performed to reveal potential mechanisms of berberine action in the treatment of patients with type 2 diabetes and dyslipidemia. Serum samples of 60 patients before and after treatment with either berberine or placebo were collected. UPLC/Q-TOFMS coupled with pattern recognition analysis was used to identify changes in global serum metabolites [130]. Compared to placebo, patients before and after berberine treatment were separated into distinct clusters as displayed by the orthogonal signal correction filtered partial least-squares discriminant analysis (OSC-PLS-DA) score plot, which indicated changes in circulating metabolites after berberine treatment. Among them, free fatty acids changed markedly. These were further quantified by UPLC combined with a single quadrupole mass spectrometer (UPLC/QMS). Significant decrease in the concentrations of 13 fatty acids was observed following berberine administration, and 10 fatty acids differed statistically from the placebo. These results suggested that berberine might play a pivotal role in the treatment of type 2 diabetes through down-regulating high level free fatty acids and that comprehensive metabonomic measurements are potentially useful for studying the mechanisms of action for TCMs.

5 Conclusion and Perspective

LC/MS and related techniques are essential to study complex systems. To analyze the diversity of compounds presented in TCMs and natural medicines, multiple chromatographic and mass spectrometric techniques are needed.

From mass spectrometry point of view, vast physical-chemical heterogeneity is contained in the TCMs, which makes it especially challenging to do a comprehensively qualitative and quantitative measurement of the TCMs and their metabolomics. A multiple ionization MS strategy was reported for the analysis of human serum extracts [131]. Chromatographic separation was interfaced inline with the atmospheric pressure ionization techniques, ESI and APCI, in both the positive (+) and negative (−) ionization modes. Furthermore, surface-based matrix assisted laser desorption/ionization (MALDI) and desorption ionization on silicon (DIOS) MS were also integrated with the separation through fraction collection and offline MS. Processing of the raw data using the XCMS software resulted in time-aligned ion features, which are defined as a unique *m*/*z* at a particular retention time. The ion feature lists obtained through LC/MS with ESI and APCI interfaces in both ionization modes were compared, and unique ion tables were generated. Nonredundant, unique ion features were defined as the mass numbers for which no mass numbers corresponding to $[M + H]^+$, $[M - H]^-$, or $[M + Na]^+$ were observed in the other ionization methods at the same retention time. Analysis of the extracted serum using ESI for both (+) and (−) ions resulted in > 90% additional unique ions detected in the (-) ESI mode. Complementing ESI with APCI analysis resulted in an additional ~20% increase in unique ions. Finally, ESI/APCI ionization was combined with fraction collection and offline-MALDI and DIOS MS. The parts of the total ion current chromatograms corresponding to the collected fractions were summarized, and the *m*/*z* lists were compiled and compared to those obtained from DIOS/MALDI spectra. It was observed that DIOS accounted for ~50% of the unique ions detected for each fraction. These results suggest that true global metabolomics requires multiple ionization techniques to address

the inherent metabolite diversity and therefore the complexity of metabolomics studies.

Ultra-high speed, high resolution multiple dimension LC and high sensitivity nanospray LC or chip LC are ideal choices to analyze complex systems. A novel, fully automatic column-switching approach combining the orthogonal selectivity of hydrophilic interaction chromatography (HILIC) and reversed-phase chromatography was developed in a metabolomics study [132]. The temporal, pharmacodynamic effects of ginsenoside Rg3 on the metabonome in urine of healthy and liver-tumor-bearing rats have been investigated. Within a total analysis time of 52 min, 5686 polar, and on the second column an additional 1808 apolar, urinary metabolite ions were detected. The administration of a single, high dose of Rg3 in *α*-cyclodextrin-based formulation led to a considerable change of the metabolic pattern in cancer rats during three days' study, leading to detection of 17 biomarker candidates including three apolar metabolites which were not retained on the HILIC column. Overall, the results suggest that the developed LC/MS strategy is a promising tool in metabolomics studies for global analysis of highly complex biosamples. It may not only increase the number of discovered biomarkers, but also consequently improve the comprehensive information on metabolic changes in a fully automatic manner.

Significantly different LC/MS responses observed for many structurally different compounds limit the use of LC/MS for quantitative determination of compounds in the TCMs and metabolite studies without using reference standard. The recently introduced nanospray ionization (NSI) technique shows not only higher response, but also comparable MS responses for different compounds [133]. A response normalized-NSI-LC-MS system was recently developed [134]. The set-up involves two HPLC systems, a chip-based NSI source and a Q-TOF mass spectrometer. One HPLC unit performs the analytical separation, while the other unit adds solvent post-column with an exact reverse of the mobile phase composition such that the final composition entering the NSI source is isocratic throughout the entire HPLC run. The data obtained from four different structural classes of compounds and their metabolites indicated that by maintaining the solvent composition unchanged across the HPLC run, the influence of the solvent environment on the ionization efficiency is minimized. In comparison to responses obtained from radiochromatograms, the response normalization modification results in nearly uniform LC-NSI-MS response for all compounds evaluated.

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液相色谱/质谱联用技术的新进展和中药研究的相关热点问题

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【摘　要】 中药的副作用较少，疗效确切且价格低廉。在中国，中药广泛应用于各种疾病的预防和治疗已经有上千年的历史。然而，中药成分的复杂性却成为其现代化和全球化进程中的瓶颈。本文综述了液相色谱/质谱联用（包括四级质谱、离子阱质谱、飞行时间质谱和杂交质谱）及数据处理技术应用于中药定性、定量研究的新进展。与此同时，本文还论述了与中药研究相关的一些热点问题，如质量控制，指纹图谱，多组分定性、定量，药物代谢动力学及代谢组学。此外，对于液相色谱/质谱联用技术应用于复杂组分研究的未来发展方向，如多重离子化质谱策略，多维液相色谱及纳升液相色谱也进行了简述。

【关键词】 中药；液相色谱/质谱联用技术

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