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Review

Gain deeper insights into traditional Chinese medicines using multidimensional chromatography combined with chemometric approaches

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ABSTRACT

Traditional Chinese medicines (TCMs) possess a rich historical background, unique theoretical framework, remarkable therapeutic efficacy, and abundant resources. However, the modernization and internationalization of TCMs have faced significant obstacles due to their diverse ingredients and unknown mechanisms. To gain deeper insights into the phytochemicals and ensure the quality control of TCMs, there is an urgent need to enhance analytical techniques. Currently, two-dimensional (2D) chromatography, which incorporates two independent separation mechanisms, demonstrates superior separation capabilities compared to the traditional one-dimensional (1D) separation system when analyzing TCMs samples. Over the past decade, new techniques have been continuously developed to gain actionable insights from complex samples. This review presents the recent advancements in the application of multidimensional chromatography for the quality evaluation of TCMs, encompassing 2D-gas chromatography (GC), 2D-liquid chromatography (LC), as well as emerging three-dimensional (3D)-GC, 3D-LC, and their associated data-processing approaches. These studies highlight the promising potential of multidimensional chromatographic separation for future phytochemical analysis. Nevertheless, the increased separation capability has resulted in higher-order data sets and greater demands for data-processing tools. Considering that multidimensional chromatography is still a relatively nascent research field, further hardware enhancements and the implementation of chemometric methods are necessary to foster its robust development.

Contents

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1. Introduction

Traditional Chinese medicines (TCMs) represent a valuable heritage of the Chinese nation and have been extensively utilized for disease treatment and prevention for thousands of years (Jiang et al., 2022; Qu et al., 2022). In the post-epidemic era, the government has increasingly supported the high-quality development of the TCMs industry, resulting in unprecedented recognition globally (Hao & Liu, 2022; Liu, 2021). However, challenges remain, particularly in the field of TCMs quality, characterized by issues such as lack of rigor, non-standardization, and incompleteness. To address these challenges and improve the evaluation of TCMs (Cao, Wang, Wei, Chen, & Han, 2021), robust analytical techniques for separating chemical constituents and identifying potentially active components are indispensable (He & Zhou, 2021). While traditional one-dimensional (1D) chromatographic technologies, such as gas chromatography-mass spectrometry (GC–MS) (Guo et al., 2022), liquid chromatography - mass spectrometry (LC-MS) (Li et al., 2022a), have been widely employed for the multi-component analysis of TCMs, and have yielded satisfactory results, they often encounter issues of co-elution and low-response when applied to highly complex TCMs samples.

To overcome these limitations, researchers have developed heart-cutting multidimensional technologies by coupling two or more chromatographic columns with different separation mechanisms. Unfortunately, these approaches only achieve further separation of a portion of the components in the samples. To address this issue, comprehensive two-dimensional gas chromatography (GC × GC), comprehensive two-dimensional liquid chromatography (LC × LC) and comprehensive two dimensional supercritical fluid chromatography (SFC × SFC) have been developed, enabling the complete separation (or near-complete separation) of all components in complex samples. With advancements in science and technology, the hardware upgrades have made two-dimensional (2D) analytical systems increasingly mature over the past decade, and even three-dimensional (3D) chromatography technologies have emerged. However, these advanced instruments have also generated more complex data structures, necessitating more sophisticated data processing algorithms. Currently, several commercial software tools, such as GC-Image and Canvas, are capable of loading data from various instrument manufacturers, including Agilent, Shimadzu, and Thermo, and provide cross-vendor data processing functions, such as visualization, analysis, and reporting for multidimensional analytical systems.

In this review, we systematically summarize the recent advancements in multidimensional gas chromatography (MDGC), multidimensional liquid chromatography (MDLC), and related chemometric methods in the field of TCMs. The review highlights the growing trend of multidimensional separation of complex TCMs samples and the increasing demand for relevant data processing algorithms.

2. MDGC in TCMs field

Essential oils, found in significant medicinal parts of TCMs such as roots of *Angelica sinensis* (Oliv.) Diels, roots of *Ligusticum chuanxiong* Hort. and roots of *Saposhnikovia divaricata* (Turcz.) Schischk. (Pelvan, Karaoğlu, Fırat, Kalyon, & Ros, 2022), are rich in secondary metabolites present in the epidermal hair, oil tubes, oil cells, and other parts of aromatic plants. These metabolites exhibit various effects, including relieving exterior syndrome, removing dampness, promoting *qi* circulation, and inducing resuscitation (Kant & Kumar, 2022). Essential oils are complex mixtures composed mainly of monoterpenes and sesquiterpenes. However, their chemical composition and content can vary due to genetic factors, climate, soil conditions, and pharmaceutical techniques. Additionally, their stability can be influenced by factors such as light, high temperature, and metal impurities, leading to oxidation, isomerization reactions, and photochromic addition reactions. Consequently, it is crucial to establish reliable and rational analytical methods for assessing the components of essential oils, thereby ensuring stable and controllable TCMs quality. This, in turn, can expedite the internationalization and modernization of TCMs.

2.1. Limitations of GC-GC analyses in TCMs field

For a considerable period, one-dimensional gas chromatography (1D-GC) has been extensively employed in the detection of complex essential oils, providing valuable information on their chemical composition. It is characterized by high separation efficiency, precision, and accuracy. However, when confronted with highly complex TCMs samples, issues such as co-eluted peaks often arise, leading to inaccurate qualitative and quantitative analysis. Although scholars have addressed these problems using multivariate resolution and multi-way calibration algorithms (de Juan & Tauler, 2021; Wu, Wang, & Yu, 2020), the limited separation space and poor resolution remain significant challenges in the analysis of complex TCMs components.

To overcome these bottlenecks, researchers have developed MDGC, which combines two or more independent columns and employs a modulator to achieve orthogonal separation. Traditional MDGC, known as heart-cutting technology (GC-GC), transfers the region of interest from the first-dimensional (^{1}D) column to the second-dimensional (^{2}D) column for further separation (Tranchida, Aloisi, & Mondello, 2022). It has been used to analyze halophenols and haloanisoles in cork bark macerates (Marsol-Vall, Ainsa, Lopez, & Ferreira, 2022). However, this technology cannot achieve the additional separation of all components in a single injection. Increasing the number of peaks of interest requires expanding the working frequency of the heart-cutting window or increasing the number of injections, which significantly extends the running time to several dozen hours or more. Clearly, GC-GC technology also faces limitations in the analysis of volatile oils from complex TCMs. Nevertheless, heart-cutting technology using

GC–MS can be applied for trace analysis of impurities or contaminants in herbs or food (David, Tienpont, Devos, Lerch, & Sandra, 2013).

2.2. Chemometrics- assisted TCMs fingerprinting by GC × GC technology

In 1991, John Phillips and his colleagues developed comprehensive two-dimensional gas chromatography (GC × GC) technology, enabling the 2D separation of all components in a sample within a single run through fast and continuous heart-cutting operations, with modulation periods shorter than the ^{1}D peak width (Adahchour et al., 2006a, Adahchour et al., 2006b). The maximum peak capacity of GC × GC is the product of the ^{1}D and ^{2}D peak capacities. Compared to 1D-GC, GC × GC offers higher sensitivity and resolution, leading to better separation of complex samples. When coupled with mass spectrometry (MS) apparatus, it enhances component separation and identification (Groger, Kafer, & Zimmermann, 2020). Additionally, different compound types tend to cluster in GC × GC fingerprints (Adahchour et al., 2006c), facilitating the separation and classification of unknown compounds. However, it is important to note that the application of GC × GC in complex TCMs samples faces obstacles such as expensive costs, data processing algorithms, and the availability of suitable software. Nevertheless, several articles utilizing GC × GC for herbal analysis can be found in databases such as PubMed, NKI, and Elsevier (Table 1).

2.2.1. Qualitative analysis

The challenges of "co-elution" and "traces" have prompted herbal analysts to focus on GC × GC technologies, which offer higher peak capacity and sensitivity for rapid separation and analysis of volatile and semi-volatile components in TCMs. As illustrated in Fig. 1A, six compounds can be effectively separated in the 2D space, whereas only three peak clusters are observed in the 1D profile. This highlights the improved separation performance achieved through careful optimization of various parameters, including modulation period, oven program, and column set. For instance, when analyzing the volatile oil from *Amomum villosum* Lour, only 36 components were detected using 1D GC, whereas GC × GC combined with time-of-flight mass spectrometry (TOF MS) enabled the detection of 83 components (Chen, Xiao, Qian, Song, & Xiang, 2021). GC × GC has also been employed to identify 303 compounds in the essential oil of *Artemisia annua* Linn. (Ma et al., 2007), separate and detect 167 components in the essential oil of fruit peels of *Citrus reticulate* "Dahongpao" from Zigong in Sichuan Province, China (Qin et al., 2013), capture the unique signature of essential oil from *Mentha x piperita* var. Italo-Mitcham (Gabetti et al., 2021), and analyze two important *Curcuma* species for their antioxidant and antibacterial activities (Jena, Ray, Sahoo, Panda, & Nayak, 2020). Moreover, the GC × GC system demonstrates sufficient capability to separate enantiomeric components. For example, an enantioselective cyclodextrin column combined with a polar polyethylene glycol capillary column successfully separated ephedrine alkaloids and their enantiomers in Ephedra raw herbs and proprietary products (Wang, Marriott, Chan, Lee, & Huie, 2006).

In the analysis of herbal formulas, which are more complex than individual herbs, 2D-GC is continuously tested for its separation capabilities. Fig. 1B demonstrates an example of overlapped peaks in the 2D space. To address this, data conversion or splitting can be applied, followed by the utilization of multivariate curve resolution-alternating least squares (MCR-ALS) based on a bilinear model, alternating trilinear decomposition (ATLD) based on a trilinear model, or even non-iterative MCR methods to resolve pure profiles in the 2D space. For instance, MCR-assisted GC × GC separation was employed to qualitatively analyze terpenoids and phthalides in Chaihu Shugan San, a formula composed of seven medicinal materials (He et al., 2017). GC × GC data in this study provided three orthogonal pieces of information, including ^{1}D and ^{2}D retention time and mass spectra, resulting in higher peak capacity and selectivity. This approach ensured more accurate quantitative analysis and enhanced the reliability of volatile component identification in TCMs. To narrow down the candidates of unassigned peaks, orthogonal information and artificial intelligence can be further considered, as shown in Fig. 1C. In one case, GC × GC separation, fragmentation rules, and retention index (RI) prediction were combined to analyze sesquiterpenoids in the essential oil of *Cyperus rotundus* L. (He et al., 2018). Another perspective is that GC × GC can be employed to identify valuable or potent herbs within complex mixtures or formulations, offering higher sensitivity. For example, *Panax quinquefolius* L. (American ginseng) can be identified from herbal mixtures using headspace solid-phase microextraction (HS-SPME) with GC × GC determination (Di, Shellie, Marriott, & Huie, 2004). In the future, chemometrics-assisted HS-SPME/GC × GC–MS can be applied for non-destructive analysis of different medicinal materials or formulas to authenticate their authenticity (Tranchida, Aloisi, & Mondello, 2020).

2.2.2. Group-type differentiation

The quality of TCMs is influenced by various factors such as genes, soil conditions, sunlight, harvest time, and pharmaceutical technologies, which affect the types and contents of secondary metabolites. Chemical pattern recognition has been widely used in research to achieve regional differentiation and variety identification, among other applications. It is an essential scientific tool that can unveil hidden information from complex herbs. Currently, a significant amount of LC or GC data has been employed for pattern recognition. However, with advancements in hardware, the utilization of 2D-GC analysis combined with pattern recognition can provide a more reasonable assessment of the quality of complex TCMs. Fig. 2 outlines the flowchart for pattern recognition or feature selection using 2D-GC or 2D-LC data. The main steps involved are as follows: data acquisition, signal pre-processing, alignment, peak identification, data pre-processing, and pattern recognition or feature selection. Pattern recognition methods typically include supervised pattern recognition, unsupervised pattern recognition cluster analysis, pattern recognition based on projection, classification, and regression trees. For example, in a study (Qiu et al., 2007), GC × GC-TOF MS and multivariable analysis methods were employed to investigate the volatile oil in the rhizomes and radixes of *Notopterygium incisum* Ting ex H.T. Chang (known as Qianghuo in Chinese) from different regions. The content of monoterpenes and oxygenated sesquiterpenes served as markers for group-type differentiation. Another study (Qiu et al., 2008) used a GC × GC system and principal component analysis (PCA) to classify root samples of *Panax ginseng* C.A. Mey. of different ages, with four components identified as quality markers. In recent years, the safety and quality of TCMs have been significantly affected by potential harmful pollutants such as heavy metals, pesticide residues, and sulfur dioxide. Sulfur fumigation is a common method used in the processing of some TCMs to prevent insects and mold and facilitate storage. However, in the industry, sulfur treatment is often overused to retain moisture, increase weight, enhance appearance, and boost sales. Although the industry is

Table 1
Application of GC × GC in herbal analysis.

Samples	Columns		Detectors	References
	^{1}D	^{2}D		
Amomum villosum (Sharen): fruits	DB-5MS (30 m × 0.25 mm, 0.25 μm)	DB-17 (1.2 m × 0.18 mm, 0.18 μm)	qTOF-MS	Chen, Xiao, Qian, Song, & Xiang, 2021
A. annua (Huanghuahao): leaves, flowers	DB-Petro (50 m × 0.20 mm, 0.5 μm)	DB-17ht (2.6 m × 0.1 mm, 0.1 μm)	TOF-MS	Ma et al., 2007
C. reticulate (Chenpi): peels	DB-XLB (15 m × 0.25 mm, 0.25 μm)	BPX-50 (SGE) (1.0 m × 0.1 mm, 0.1 μm)	HR-TOFMS	Qin et al., 2013
M. piperita: leaves, stems	DB-5 (30 m × 0.25 mm, 0.25 μm)	OV1701 (2.0 m × 0.1 mm, 0.1 μm)	TOF-MS	Gabetti et al., 2021
Mentha haplocalyx Briq (Bohe): leaves, stems	DB-XLB (30 m × 0.25 mm,0.25 μm)	BPX-50 (SGE) (1.0 m × 0.1 mm, 0.1 μm)	TOF-MS	Cao et al., 2011
M. haplocalyx (Bohe): leaves, stems	HP-5MS (30 m × 0.25 mm, 0.25 μm)	DB-17HT (30 m × 0.10 mm, 0.1 μm)	TOF-MS	Yan et al., 2023
Atractylodes macrocephala Koidz. (Baizhu): roots	DB-5MS (30 m × 0.25 mm, 0.25 μm)	DB-1701 (2.0 m × 0.1 mm, 0.1 μm)	TOF-MS	Cao et al., 2014
Angelica pubescens Maxim. f. biserrata Shan et Yuan (Duhuo): roots	HP-5MS (30 m × 0.25 mm, 0.25 μm)	DB-17MS (1.0 m × 0.18 mm, 0.18 μm)	qTOF-MS	Qian et al., 2022
Zedoary volatile oil	SOLGELWAX (60 m × 0.25 mm, 0.25 μm)	Cyclodex-B (3.0 m × 0.1 mm, 0.1 μm)	TOF-MS	Wu et al., 2004
Curcuma species (*C. angustifolia* and *C. zedoaria*)	Rxi-5Sil MS (30 m × 0.25 mm, 0.25 μm)	Rxi-17Sil MS (2.0 m × 0.25 mm, 0.25 μm)	TOF-MS	Jena, Ray, Sahoo, Panda, & Nayak, 2020
Ephedra (Mahuang): stems	Cyclodex-B (30 m × 0.25 mm, 0.25 μm)	BP20 (1.0 m × 0.1 mm, 0.1 μm)	FID	Wang, Marriott, Chan, Lee, & Huie, 2006
P. quinquefolius (Huaqishen): roots	HP-5 (30 m × 0.32 mm, 0.25 μm)	BP20 (1.0 m × 0.1 mm, 0.1 μm)	FID	Di, Shellie, Marriott, & Huie, 2004
C. rotundus (Xiangfu): roots	Rtx-5Sil MS (30 m × 0.25 mm, 0.25 μm)	BPX-50 (2.5 m × 0.1 mm, 0.1 μm)	qMS	He et al., 2018
N. incisum (Qianghuo): roots	CEC-1 (50 m × 0.25 mm, 0.25 μm)	DB-WAX (1.8 m × 0.1 mm, 0.1 μm)	TOF-MS	Qiu et al., 2007
P. ginseng (Renshen): roots	DB-5MS (30 m × 0.25 mm, 0.25 μm)	DB-1701 (1.6 m × 0.1 mm, 0.1 μm)	TOF-MS, FID	Qiu et al., 2008
Chrysanthemum × *morifolium* (Juhua): flowers	DB-5MS (30 m × 0.25 mm, 0.25 μm)	DB-17ht (2.0 m × 0.1 mm, 0.1 μm)	TOF-MS	Cao et al., 2012
L. Japonicae (Jinyinhua): flowers	DB-5MS (30 m × 0.25 mm, 0.25 μm)	DB-17ht (2.0 m × 0.1 mm, 0.1 μm)	TOF-MS	Cai et al., 2013
A. sinensis (Danggui): roots	DB-5MS (30 m × 0.25 mm, 0.25 μm)	DB-1701 (1.0 m × 0.1 mm, 0.1 μm)	HR-TOF-MS	Cai, Cao, & Zhang, 2017
	DB-WAX (60 m × 0.25 mm, 0.25 μm)	DB-17MS (1.0 m × 0.15 mm, 0.15 μm)	TOF-MS	Zhang et al., 2023
Chaihu Shugan San	Rtx-5Sil MS (30 m × 0.25 mm, 0.25 μm)	BPX-50 (2.5 m × 0.1 mm, 0.1 μm)	qMS, TOF-MS	He et al., 2017; He, Zeng, Peng, Zhou, & Cao, 2021
Chaihu Shugan Wan	DB-WAX (30 m × 0.25 mm, 0.25 μm)	DB-17MS (1.195 m × 0.15 mm, 0.15 μm)	TOF-MS	He et al., 2023

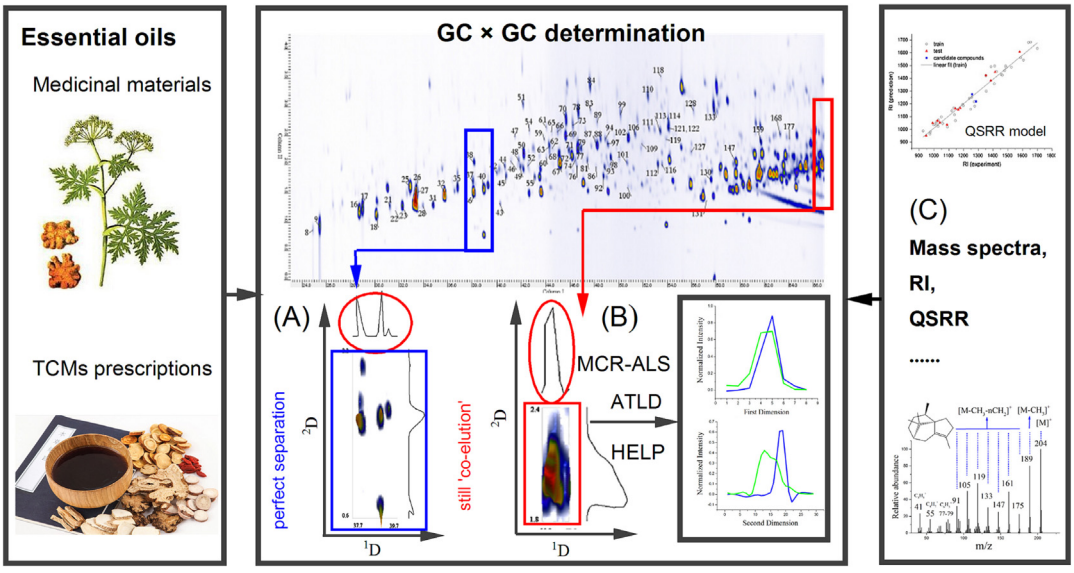

Fig. 1. (A) Separation of six phytochemicals in 2D space, compared to three peak clusters in 1D profile; (B) Development of chemometric methods to address co-eluted peaks; (C) Multi-analytical strategy for unassigned peaks in TCMs samples using fragmental rules and RI prediction, among others.

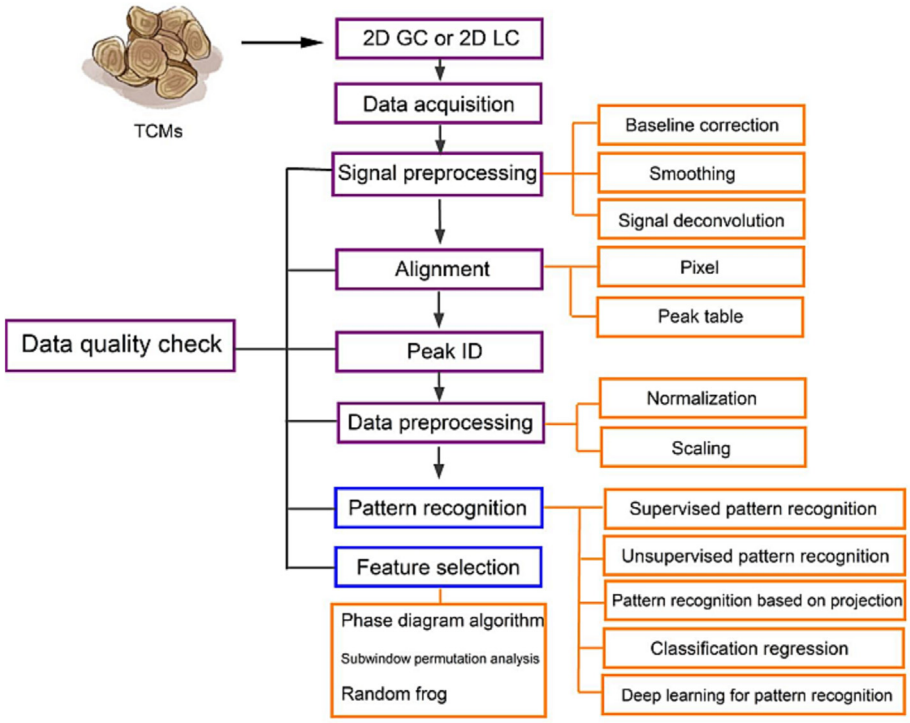

Fig. 2. Flowchart illustrating pattern recognition or feature selection for 2D GC data.

gradually promoting "sulfur-free" processing technology, fresh processing, new drying methods, and storage techniques, excessive or abusive sulfur fumigation still persist. Researchers have utilized GC × GC-TOF MS to differentiate sun-dried herbal samples from sulfur fumigated ones and successfully identified markers to distinguish between them (Cao et al., 2012; Cai et al., 2013). Additionally, through GC × GC and the color fuzzy difference method, the scholars (Cai, Cao, & Zhang, 2017) discovered that 25 compounds were absent in sulfur-fumigated *Angelicae Sinensis Radix* samples compared to dried samples, and 17 volatile components were observed in the sulfur-fumigated samples for the first time.

2.2.3. Pairwise comparison

Comparing complex samples within or between groups is often challenging in GC × GC separation and analysis. Various factors such as genes, climate, soil conditions, processing, and storage can influence certain components in TCMs samples. These components can serve as important markers for differentiating dozens or even hundreds of samples. However, the abundance of information and large data storage in GC × GC analysis can be overwhelming for data analysts. To address this issue, Multi-component Spectral Correlative Chromatography (MSCC) has been employed to assess the correlation between different groups of GC × GC-TOF MS data and identify relevant markers. It is important to note that ^{2}D peaks are formed by the continuous modulation of the same or different ^{1}D peaks, resulting in intricate patterns. Considering the computational burden of including all modulation peaks in the MSCC calculation, comparing the entire dataset becomes cumbersome. Conversely, manual recognition that involves comparing peaks one by one using coordinates and mass spectra is time-consuming and labor-intensive. To overcome these challenges, scholars have proposed an intelligent clustering of modulation peaks - the Intelligent Clustering of Modulation Peaks-Multi-component Spectral Correlation Chromatography (ICMP-MSCC) algorithm. This algorithm includes restrictions on ^{2}D peak selection, ^{1}D peak shape restriction, calculation of eigenvalues against mass spectra in moving sub-windows, and MSCC calculation. In Fig. 3, the ICMP-MSCC algorithm was used to compare the similarities (represented by 'o') and differences (represented by red 'x') of each peak group in two GC × GC-TOF MS or GC × GC-qMS fingerprints obtained from Chaihu Shugan formula or *Cyperus rotundus* essential oils (He, Zeng, Peng, Zhou, & Cao, 2021). In another case, ICMP-MSCC was applied to distinguish the inter-group and intra-group differences in Chaihu Shugan Wan (CSW). Furthermore, an image similarity strategy based on zone- specific ion filtration was proposed for rapidly evaluating 2D fingerprints of Chinese patent medicines (He et al., 2023).

2.3. Prospects for 3D-GC technologies

The characterization of TCMs samples requires extremely high separation efficacy. While GC × GC separation has been developed, it is still insufficient for the complexity of TCMs prescriptions. This demand for higher peak capacities has led to the development of 3D gas chromatography (GC × GC × GC), which utilizes two modulators connected to three analytical columns to achieve a narrow enough chromatographic bandwidth for 3D separation. The first reported 3D-GC system in 2000 employed a new thermal modulation scheme with a single moving heater to operate two thermal modulators and connect three separation columns (Ledford, Billesbach, & Zhu, 2000), which is the first reported 3D-GC system. In current systems, two six-port diaphragm valves are used as modulators between three in-series capillary columns within a standard GC instrument, resulting in significantly higher peak capacity (Siegler, Crank, Armstrong, & Synovec, 2010). To improve peak identification, researchers have used Time-of-Flight Mass Spectrometry (ToF MS) instead of a flame ionization detector (FID) and adopted high-temperature diaphragm valves to expand the operational temperature range (Watson, Bahaghighat, Cui, & Synovec, 2017). In 2018, scholars further enhanced the peak capacity of 3D-GC through pulse flow valve modulation or column selection (Gough, Bahaghighat, & Synovec, 2019; Gough, Song,

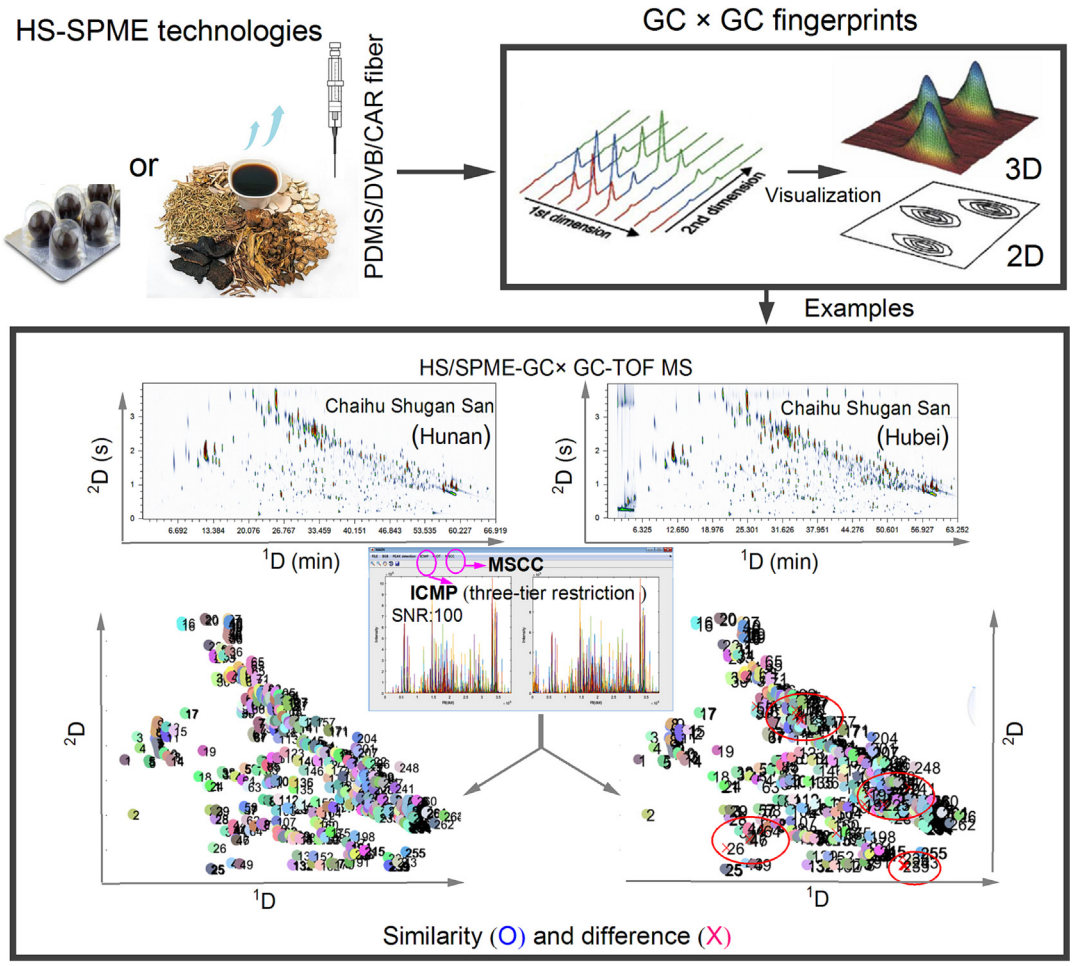

Fig. 3. ICMP-MSCC result for two sets of HS/SPME-GC × GC -TOF MS data.

Schöneich, Prebihalo, & Synovec, 2019). With the rapid development of 3D-GC instruments, peak capacity and selectivity are expected to improve even further in the future. This advancement in peak capacity and selectivity will enable 3D-GC to excel in the analysis of complex samples (Abdulhussain, Nawada, & Schoenmakers, 2021). A positive development was demonstrated in the separation and detection of oxygenated sesquiterpenes in hop (*Humulus lupulus* L.) essential oil and agarwood (*Aquilaria malaccensis* Lam.) oleoresin, where improved resolution and peak capacity of sequential hybrid 3D-GC were achieved (Yan et al., 2018).

Different multi-way calibrations have been applied to high-order analytical data based on the types of instrument signals. The Synovec group, for example, applied a four-way PARAFAC method to analyze 3D-GC-TOFMS data, which allows for the extraction of more inherent information from complex TCMs samples and exhibits the "third-order advantage" (Watson, Prebihalo, & Synovec, 2017). The effects of other third-order calibration algorithms such as Alternating Quadrilinear Decomposition (AQLD) remain to be explored. Furthermore, there is a need for robust and user-friendly software to visualize and interpret 3D-GC datasets. Fisher ratio software, for instance, has been applied to 3D-GC data, treating the third chromatographic dimension (^{3}D) as the "spectral" dimension (Trinklein, Prebihalo, Warren, Ochoa, & Synovec, 2020). In another case, the analytical utility of non-targeted chemometric analysis was demonstrated on 3D-GC-TOFMS data using principal component analysis (PCA) (Sudol, Schöneich, & Synovec, 2022). This approach introduced a 2D re-registration method prior to PCA to center the data while preserving the chemical class structure of the separations, resulting in more interpretable and informative data. From the literature mentioned above, it is evident that 3D-GC data is complex and requires further exploration of new chemometric methods. Particularly in the field of TCMs, more applications will impose higher requirements for data processing in the future.

3. MDLC in TCMs field

Bioactive compounds in TCMs such as flavonoids (Qin et al., 2022), saponins (Zhang, Ying, Zhao, Chen, & Deng, 2022), alkaloids (Feng, Ju, Chen, Li, & Wang, 2022), diterpenoids (Hou, Yao, & Song, 2021), and polysaccharides (Guo et al., 2022) are mostly non-volatile and require appropriate separation methods using liquid chromatography (LC). In many cases, conventional 1D-LC is not sufficient to fully separate target components in one run due to its limited peak capacity and separation efficiency, which is insufficient for the analysis of complex TCMs. As a result, 2D-LC, which is based on two independent separation mechanisms, has been developed (Foster et al., 2022). Various 2D-LC configurations have been utilized, including reverse phase LC (RPLC) × RPLC, normal phase LC (NPLC) × RPLC, hydrophilic interaction LC (HILIC) × RPLC, size exclusion chromatography (SEC) × RPLC, and ion exchange chromatography (IEC) × RPLC. Due to its high peak

capacity and selectivity, 2D-LC has found wide applications in the fields of food, pharmaceuticals, toxicology, and TCMs, yielding significant results (Cacciola, Rigano, Dugo, & Mondello, 2020; Cao et al., 2014; Iguiniz & Heinisch, 2017; Xiao, Jian, & Li, 2014; Ji et al., 2018; Zhou et al., 2020).

From an application perspective, 2D-LC can be categorized into off-line mode and on-line mode, depending on the presence of a modulator between the ^{1}D and ^{2}D columns. In the off-line mode, the effluent from the ^{1}D column is typically concentrated and re-injected into the ^{2}D column, making it suitable for compound purification from medicinal materials. On the other hand, on-line 2D-LC is more demanding and challenging but offers high reproducibility and automation, making it suitable for high-throughput analysis of TCMs. On-line 2D-LC can be further divided into heart-cutting liquid chromatography (LC-LC) and comprehensive two-dimensional liquid chromatography (LC × LC), depending on whether all components from the ^{1}D column are transferred to ^{2}D column. In heart-cutting LC-LC, only selected components containing the target analytes are transferred to the ^{2}D column, which is suitable for the separation and purification of specific compounds. In comprehensive LC × LC, all components are fully separated in two dimensions, allowing for non-targeted analysis of complex TCMs to obtain as much information as possible.

3.1. Chemometrics assisted off-line MDLC analyses

3.1.1. Off-line 2D-LC separation and identification of TCMs components

Compared to 1D separation, off-line 2D-LC significantly increases peak capacity, facilitating the identification of trace components. This method eliminates solvent incompatibility issues and allows for flexible selection of organic solvents. However, it should be noted that manual collection / concentration of components of interest from the ^{1}D column to the ^{2}D column may introduce risks of degradation or loss. Moreover, the drawbacks of poor reproducibility and time-consuming procedures have limited the development of off-line 2D-LC technology. Nonetheless, it remains applicable in quality and material basis studies due to its ability to separate complex TCMs samples. The workflow of the off-line 2D LC system is outlined clearly in Fig. 4. Only with the help of a series of chemometric approaches can the data be interpreted more accurately. Notoginsenosides, *Ginkgo biloba* L. extract, Qingkailin Injection, roots of *Scutellaria baicalensis* Georgi and the dried unripe fruit of *Citrus aurantium* L. have been analyzed using off-line 2D-LC, and the results can be found in the literature published before 2020 (Cao et al., 2014; Ji et al., 2018; Zhou et al., 2020). Additional literature on herbal analysis since 2020 can be obtained from PubMed, Willy, and Elsevier databases, as shown in Table 2.

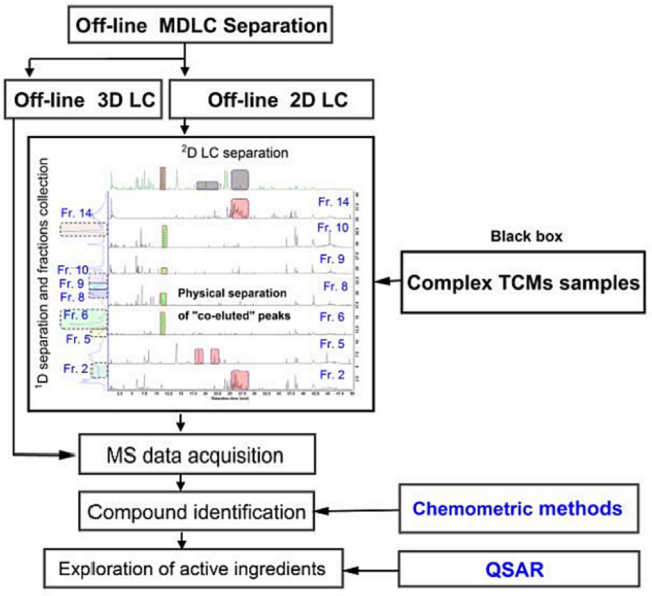

Fig. 4. Flowchart demonstrating separation and identification of phytochemicals using an off-line MDLC approach.

Over the past three years, the off-line 2D-LC method has been developed not only for the separation and analysis of medicinal material extracts (Lecas, Nuccio, De Vaumas, & Faure, 2021) but also for the identification of components in Chinese herb prescriptions (Shen et al., 2022) and quality control of TCMs. For instance, off-line 2D-LC-MS technology combined with automatic peak annotation, molecular networking, and collision cross-section (CCS) prediction has identified or tentatively characterized a total of 302 compounds in *Cuscuta chinensis* Lam. (Wang et al., 2022c). In another study (the middle frame of Fig. 4), off-line 2D LC-MS, along with a novel mixed MS scanning method, was employed to identify 403 components in Compound Danshen Dripping Pill (Wang et al., 2022a). These examples highlight the crucial role of MS technology and related chemometric methods in the separation and identification of TCMs components. Notably, similar MS-processing methods (e.g., ionic isotopes optimization, fragment rules, and mass spectrum prediction) can also be applied to 2D-LC analyses (Hong et al., 2020; Zeng, Wu, & He, 2023; Zeng et al., 2021; Zhang et al., 2022), and machine learning and other chemometric methods can indirectly improve component identification in 2D chromatography.

From a drug target perspective, off-line 2D-LC-MS can also be utilized to explore active ingredients in TCMs or functional foods. For instance, the experts (Cheng, Kong, Li, & Li, 2022) employed off-line 2D-LC-MS in combination with affinity screening and molecular docking to identify 5-LOX/COX-2 dual target inhibitors in *Oroxylum indicum* L. extract. Virtual screening approaches such as machine learning and artificial intelligence have been applied to quantitative structure–activity relationship (QSAR) studies (Bitam, Hamadache, & Hanini, 2022; Lin & Chou, 2022), contributing to 2D-LC-MS research. Researchers believe that the chemical structure plays a pivotal role in determining the biological activity of phytochemical molecules, and thus, QSAR mathematical models can be established. *In silico* chemical-protein interactions (CPIs) are a crucial area of research in TCMs, especially in the field of herbal quality control. Numerous servers, including STITCH 5.0, DRAR-CPI, COPICAT, and PharmMapper, have been implemented for this purpose. Various algorithms based on random forest, support vector machine, and deep learning have been developed for molecular modeling. By combining these approaches, 2D-LC technology can be further employed for screening active components in TCMs samples.

3.1.2. Off-line 3D-LC separation of TCM components

TCMs are characterized by complex chemical systems containing a wide range of primary and secondary metabolites with varying acid-base properties, polarity, molecular weight, and contents. The characterization of these metabolites in TCMs samples presents a significant challenge. To address this issue, a three-dimensional liquid chromatography (3D-LC) system, combining ion exchange chromatography (IEC), hydrophilic interaction chromatography (HILIC), and reversed-phase chromatography (RPC) (IEC-HILIC-RPC), was established to characterize the active components of *Uncaria sessilifructus* Roxb. This system successfully separated 308 components, and 128 components were identified or tentatively characterized, demonstrating superiority over conventional 1D LC/MS methods (Feng et al., 2021). Furthermore, off-line 3D-LC/MS allows for the flexible configuration of multiple

Table 2
Application of off-line MD-LC in herbal analysis.

Samples	Columns	Detectors	References
Polygonum cuspidatum Sieb. et Zucc (Huzhang): roots	^{1}D: Countercurrent chromatography ^{2}D: Phenomenex Luna C_{18} (150 mm × 4.60 mm, 5 μm)	UV, ESI-MS	Wang et al., 2020b
	^{1}D: Countercurrent chromatography ^{2}D: HE-C_{18} (250 mm × 4.6 mm, 5 μm)	UV	Xu et al., 2022
Uncariae Ramulus cum Uncis (Gouteng): stems	^{1}D: Waters XBridge Amide (4.6 mm × 150 mm, 3.5 μm) ^{2}D: Phenomenex Kinetex EVO C_{18} (2.1 mm × 100 mm, 2.6 μm)	Q-TOF-MS	Li et al., 2021
U. sessilifructus: stems	^{1}D: PhenoSphere™ SCX (4.6 mm × 250 mm, 5 μm) ^{2}D: Acchrom XAmide (4.6 mm × 150 mm, 5 μm) ^{3}D: CSH Phenyl-Hexyl (2.1 mm × 100 mm, 1.7 μm)	Q-Orbitrap-MS	Feng et al., 2021
P. ginseng, *P. notoginseng*: flowers, buds	^{1}D: PhenoSphere SAX (4.6 mm × 250 mm, 5 μm) ^{2}D: XBridge Amide (4.6 mm × 150 mm, 3.5 μm) ^{3}D: BEH Shield RP_{18} (2.1 mm × 100 mm, 1.7 μm)	Q-Orbitrap-MS	Jia et al., 2022
Saponins from *P. notoginseng*	^{1}D: Countercurrent chromatography ^{2}D: Agilent TC-C_{18} (250 mm × 4.6 mm, 5 μm)	Dual wavelength detector	Sun, Zhang, Bao, Chu, & Tong, 2022
Steviol glycosides in *Stevia rebaudiana* Bertoni: leaves	^{1}D: C_{18} (20 mm × 250 mm, 10 μm) ^{2}D: PA (4.6 mm × 250 mm, 5 μm)	UV	Wang et al., 2022b
Two *Astragalus species*: roots	^{1}D: XAmide (4.6 mm × 150 mm, 5 μm) ^{2}D: CSH Fluoro-Phenyl (2.1 mm × 100 mm, 1.8 μm) ^{3}D: Cosmocore C_{18} (2.1 mm × 100 mm, 2.6 μm)	UV; Q-TOF-MS	Zhao et al., 2023a
Sanguisorba officinalis L. (Diyu): roots	^{1}D: M−PVS (150 mm × 4.6 mm, 5 μm) ^{2}D: Brownlee spp C_{18} (100 mm × 2.1 mm, 2.7 μm)	Q-TOF-MS	Dai et al., 2022
Glycosides from *Hedyotis diffusa* Willd.	^{1}D: C_{18} HCE (4.6 mm × 250 mm, 5 μm) ^{2}D: HILIC	UV	Dai et al., 2023
Safflower (Honghua): flowers	^{1}D: TAC column ^{2}D: Shim-pack VP-ODS (10 mm × 2.0 mm, 5 μm)	ESI-MS	Qiao et al., 2021
Homoisoflavonoids from *Ophiopogon japonicus* (L. f.) Ker Gawl. (Maidong): roots	^{1}D: HSCCC ^{2}D: SunFire™ C_{18} (4.6 mm × 250 mm, 5 μm)	DAD; Q-TOF-MS	Deng, Xu, Tong, Shi, & Shi, 2020
Qingfei Paidu Decoction	^{1}D: C_{18}YE column (50 mm × 250 mm, 10 μm) ^{2}D: BEH Amide (2.1 mm × 150 mm, 1.7 μm)	UV; Q-TOF-MS	Shen et al., 2022
C. chinensis (Tusizi): seeds	^{1}D: Waters XBridge Amide (4.6 mm × 150 mm, 3.5 μm) ^{2}D: Agilent Zorbax SB-Aq (2.1 mm × 100 mm, 1.8 μm)	Q-TOF-MS	Wang et al., 2022c
Compound Danshen Dripping Pill	^{1}D: XBridge Amide (4.6 mm × 150 mm, 3.5 μm) ^{2}D: HSS T3 (2.1 mm × 100 mm, 1.8 μm)	Q-TOF-MS	Wang et al., 2022a
Huanglian Jiedu Decoction	^{1}D: Waters Xbridge HILIC column ^{2}D: Waters Acquity HSS T3 column	Q-Exactive Orbitrap-MS	Li, et al., 2022b
O. indicum	^{1}D: Oasis HLB (2.1 mm × 20 mm, 25 μm) ^{2}D: Kinetex C_{18} (4.60 mm × 100 mm, 2.6 μm)	DAD; ESI-MS	Cheng, Kong, Li, & Li, 2022

chromatographic techniques to meet the requirements of different complex separation systems. For example, ginsenosides, important bioactive components in *Panax* plants with significant tonic effects, exhibit a wide range of acid-base properties (neutral saponins and acidic saponins) and have a low molecular weight (400–1400 Da). This poses challenges in the identification of new saponins using traditional methods. To address this, an off-line 3D-LC/Q-Orbitrap-MS analysis method was proposed. The total extract was separated into neutral (unreserved) and acidic (retained) components using ion exchange chromatography (IEC) on a PhenoSphereTM SAX column. Subsequently, hydrophilic and hydrophobic separations were achieved using HILIC (XBridge Amide column) and RPC (BEH Shield RP18 column), respectively. Finally, the system was connected to Orbitrap MS for detection. This approach was successfully applied to analyze 803, 795, and 833 types of ginsenosides from *P. ginseng*, *P. quinquefolius*, and *Panax notoginseng* (Burk) F. H. Chen flower buds, respectively (Jia et al., 2022). The results demonstrated that the 3D-LC method has great potential in the comprehensive characterization of complex TCMs samples and can be utilized in the discovery of new lead compounds.

3.2. On-line MDLC analysis of TCMs

On-line 2D-LC technology, although more demanding and challenging, offers high reproducibility and automation, making it suitable for high-throughput analysis of TCMs. It can be further categorized into LC-LC and LC × LC, depending on whether the components from the ^{1}D column are fully transferred to the ^{2}D column. LC-LC is applicable only to target separation, as it transfers only the selected components of interest to the ^{2}D column. On the other hand, LC × LC enables the complete separation of the entire sample in two dimensions, making it suitable for non-targeted analysis of complex TCMs to obtain as much information as possible (Zhou et al., 2020). Currently, both LC-LC and LC × LC technologies have been applied in the field of TCMs.

3.2.1. LC-LC system in TCMs field

LC-LC, which connects the ^{1}D column and the ^{2}D column with a special modulator, is widely used in the analysis of TCMs. The most commonly used modulator is an on–off valve controlled by an electronic device, which automates the transfer of components of interest from the ^{1}D column to the ^{2}D column. Compared to off-

line 2D-LC, this automatic system offers advantages such as shorter analysis time and better reproducibility.

Compared to 1D-LC systems, on-line LC-LC systems exhibit higher efficiency, lower solvent consumption, and shorter analysis time. They are particularly suitable for screening and separating specific types of compounds or interested components in TCMs. For instance, on-line 2D-LC separation and analysis of ginsenosides and coumarins have been reported in the literature prior to 2020 (Cao et al., 2014; Ji et al., 2018; Zhou et al., 2020). Continuous techniques like these yield higher productivities compared to traditional approaches and are effective in the separation of chemical components in TCMs. However, on-line LC-LC systems face challenges when separating TCMs components with similar structures but different polarities due to 1D bandwidth broadening. Researchers have successfully developed on-line stop-flow heart-cutting 2D-LC systems with column switching and back-flush to address this issue, allowing the separation of 12 components in *Fagopyrum tataricum* (L.) Gaertn. with similar structures but different polarities (Ren, Wu, & Zhang, 2013). Additionally, traditional LC-LC systems may encounter the problem of 2D solvent incompatibility. To overcome this, the analysts used a vacuum-evaporation loop-type valve modulator to construct an on-line 2D-NP/RPLC system, which not only resolved the issue of 2D solvent incompatibility but also enhanced chromatographic performance by reducing the spectral bandwidth of the primary fraction injected into the ^{2}D column. This method was applied to the separation and identification of 16 polycyclic aromatic hydrocarbon mixtures and *Angelica dahurica* (Baizhi in Chinese) samples (Tian, Xu, Xu, & Guan, 2006). In Table 3, numerous articles since 2019 that utilize LC-LC for herbal analysis can be found in databases such as PubMed, Wiley and Elsevier.

Targeted isolation of free radical inhibitors from natural product extracts plays a vital role in exploring the relationship between oxidative stress and various human diseases. For example, the extract of the Tibetan medicine *Ribes himalense* Royle ex Decne. was prepared using medium pressure chromatography, and two DPPH inhibitors (purity > 95 %) were subsequently separated using a 2D RPLC/RPLC combination with on-line HPLC-DPPH detection (Liu et al., 2021). *Saxifraga atrata* Engl., another Tibetan medicine, has also been found to possess antioxidant effects. The scholars purified and separated active compounds using RP-C_{18} and HILIC columns, resulting in the isolation of four high-purity free radical inhibitors (Dawa et al., 2021). Similarly, other researchers have employed on-line LC-LC systems to obtain high-purity DPPH inhibitors through targeted separation of *Saxifraga tangutica* Engl. (Dang et al., 2021 a), *Saxifraga sinomontana* J. T. Pan et Gornall (Dang et al., 2021b) and *S. atrata* (Fang et al., 2022). In another study, a PLE/2D-CCC on-line system combined with a PC12 cell model was used to separate high-purity acetylcholinesterase inhibitors, leading to the identification and isolation of six major compounds from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) (Li, Liu, Zhang, & Tsao, 2021). The heart-cutting LC-LC system has also been applied to the separation of isomers in the TCMs field. For example, researchers developed a heart-cutting 2D LC-HRMS method to improve the separation of isoflavones and their glycosides, achieving a reasonable total operation time (<25 min) and good repeatability (RSD < 2 %) (Pua et al., 2021). These results demonstrate that target compounds can be screened, isolated, and purified from various natural products using LC-LC.

On-line LC-LC also enables accurate quantitative analysis. For instance, it has been used to determine the content of chlorogenic acid and cynaroside in flower of *Lonicerae Japonicae* Thunb (*Lonicerae Flos*) with improved separation of co-eluted peaks in the 1D mode, and the high-resolution sampling mode enhances the quantitative capability of 2D-LC (Wang, Fu, Ji, & Chen, 2019). In another study, a 2D-LC/IT-TOF MS system was utilized to separate and characterize five impurities in Rutin Tablets, thereby improving the quality control of the tablets (Wang et al., 2020a). Some researchers established an on-line central cleavage LC-LC-DAD-ESI-MS method for the quantitative analysis of C-glycosylflavones in *fenugreek seeds* (*Foenugraeci Semen*, *Trigonella foenum-graecum* L.) extracts (Krol-Kogus, Glod, Halasa, & Krauze-Baranowska, 2021). Furthermore, LC-LC can contribute to quality control in TCMs by evaluating the impact of different technologies on the components of TCMs. Scholars employed a newly developed heart-cutting 2D-LC-HRMS method to investigate the effect of different drying methods on herbal components, providing a scientific

Table 3
Application of LC-LC in herbal analysis.

Samples	Columns		Detectors	References
	^{1}D	^{2}D		
R. himalense: leaves, stems	PTAS (20 mm × 250 mm, 7 μm)	ReproSil-Pur C_{18} AQ (20 mm × 250 mm, 5 μm)	UV	Liu et al., 2021
S. atrata: whole herb	ReproSil-Pur C_{18} AQ (4.6 mm × 250 mm, 5 μm)	ReprpSil-Pur C_{18} AQ (20 mm × 250 mm, 5 μm)	UV; MS	Dawa et al., 2021; Fang et al., 2022
S. tangutica: whole herb	ReproSil-Pur C_{18} AQ (4.6 mm × 250 mm, 5 μm)	ReproSil-Pur C_{18}AQ (20 mm × 250 mm, 5 μm)	UV; Q-TOF-MS	Dang et al., 2021a
S. sinomontana: whole herb	ReproSil-Pur C_{18} AQ (4.6 mm × 250 mm, 5 μm)	ReproSil-Pur C_{18} AQ (20 mm × 250 mm, 5 μm)	UV; MS	Dang et al., 2021b
A. membranaceus (Huangqi): roots	2D-CCC	C_{18} column (250 mm × 4.6 mm, 5 μm)	MS	Li, Liu, Zhang, & Tsao, 2021
Isomeric flavonoids and their glycosides	Poroshell 120 EC-C_{18} (300 mm × 100 mm, 2.7 μm)	ACQUITY UPLC HSS PFP (3.0 mm × 100 mm, 1.8 μm)	MS	Pua et al., 2021
L. Japonicae (Jinyinhua): flowers	ZORBAX RRHD Eclipse Plus C_{18} (100 mm × 2.1 mm, 1.8 μm)	ZORBAX RRHD SB-Phenyl (50 mm × 3.0 mm, 1.8 μm)	UV	Wang, Fu, Ji, & Chen, 2019
Rutin Tablets	Thermo Acclaim 120™ C_{18} (4.6 mm × 250 mm, 5 μm)	Shimadzu Shim-pack GISS C_{18} (50 mm × 2.1 mm, 1.9 μm)	MS	Wang et al., 2020a
T. foenum-graecum: seeds	Kinetex C_{18} (100 mm × 2.1 mm, 2.6 μm)	Kinetex C_{18} (100 mm × 4.6 mm, 2.6 μm)	MS	Krol-Kogus, Glod, Halasa, & Krauze-Baranowska, 2021
Leaves of *P. notoginseng*	ACQUITY UPLC BEH C_{18} (150 mm × 2.1 mm,1.7 μm)	XBridge amide (150 mm × 2.1 mm, 2.5 μm)	Orbitrap-MS	Ma, Ma, Cao, & Wan, 2022
S. tangutica	ReproSil-Pur C_{18} AQ (4.6 mm × 250 mm, 5 μm)	ReproSil-Pur C_{18} AQ (20 mm × 250 mm, 5 μm)	UV	Dang et al., 2021a
Compound Nanxing Zhitong plaster	Waters XBridge BEH C_{18} (2.1 mm × 50 mm, 1.7 μm)	Waters XBridge BEH C_{18} (3.0 mm × 100 mm, 1.7 μm)	UV	Li et al., 2019

basis for designing appropriate techniques to ensure the quality of *P. notoginseng* leaves (Ma, Ma, Cao, & Wan, 2022). Quantitative measurement of active components is essential for ensuring the effectiveness and safety of TCMs. An on-line 2D-LC method was established for the quantitative analysis of three double-ester alkaloids (neoaconitine, aconitine, and hypaconitine) in a preparation, leading to an improved quality level of TCMs products (Li et al., 2019). These studies demonstrated that the LC-LC system is an important tool for achieving quality control in TCMs.

3.2.2. LC × LC fingerprinting - overcoming challenges

Compared to off-line 2D LC or on-line LC-LC technologies, LC × LC offers higher separation capability by separating all the components in two dimensions. This system utilizes a modulator that combines two different separation processes, enabling the continuous collection of distillates from the ^{1}D column and subsequent re-injection into the ^{2}D column in the form of a pulse. The most common modulator is an on–off valve coupled to two identical storage circuits. Alternatively, a parallel column modulator can be used on a 2D connection to reduce the dead time of the ^{2}D analysis. Previous studies (Cao et al., 2014; Ji et al., 2018; Zhou et al., 2020) have utilized LC × LC apparatus for analyzing extracts from *F. lonicerae*, *L. chuanxiong*, and *Cnidii Fructus* (fruits of *Cnidium monnieri* (L.) Cuss.). Additionally, Table 4 shows that several literature references since 2020 employing LC × LC for herbal analysis can be found in PubMed, Wiley, and Elsevier databases.

Due to its strong separation ability, LC × LC is primarily focused on the chemical fingerprinting of TCMs. This focus can be attributed to two factors: (1) the high orthogonality resulting from the interconnection of hydrophilic interaction liquid chromatography (HILIC) and reversed-phase liquid chromatography (RPLC), which leads to complementary separation mechanisms and good separation. Specific reports include *Salvia miltiorrhiza* Bge. roots (Cao et al., 2018), ginsenosides in *P. notoginseng* leaves (Cao et al., 2019), ginsenosides of *P. notoginseng* inflorescence samples (Ma et al., 2020), chemical constituents from five parts of the *Buddleja davidii* Franch (Chen, Montero, Luo, Li, & Schmitz, 2020) and *P. ginseng* root extract in Fig. 5 (Chen, Li, & Schmitz, 2019). Moreover, (2) it is worth noting that, on-line HILIC × RPLC or RPLC × HILIC systems also have limitations. The solvent strengths in the two dimensions are inconsistent, resulting in solvent incompatibility between phases. This greatly affects peak shape, separation efficiency, and sensitivity in the 2D dimension. However, coupling similar columns can effectively address this issue. For instance, an on-line high-pH RPLC × low-pH RPLC-MS system was constructed to identify 39 alkaloids in *Macleaya cordata* (Willd.) R. Br. (Hu et al., 2020). In general, the incompatibility of mobile phases, dilution effect, and long run-time are the main limitations of traditional LC × LC methods. To overcome these challenges, a time-decoupled comprehensive two-dimensional ultra-high liquid chromatography coupled with an ion mobility-high resolution mass spectrometer (2D-UHPLC-IM-MS) method was established and utilized to analyze ginsenosides from the roots of white ginseng and red ginseng (Zhang et al., 2019). The combination of HILIC × HILIC is also a powerful tool for the separation and characterization of complex matrices in TCMs, as demonstrated by the analysis of safflower (*Carthamus tinctorius* L.) (Wang et al., 2021). LC × LC not only provides comprehensive characterization of herbs or their extracts but can also analyze more herbal formulations and reflect the synergistic effects of multiple components in TCMs. Researchers have established an on-line ion-exchange chromatography × RPLC (IEC × RPLC) system, used for the separation of the medicinal preparation of *Coptis chinensis* Franch (Zeng, Shao, & Fang, 2011). Similarly, a 3D fingerprint of Niuhuang Shangqing Pill was established using IEC × RPLC with different separation mechanisms, resulting in 60 more peaks compared to the traditional 1D-LC method (Wu et al., 2019). This provides a more comprehensive material composition and macroscopic information for quality control of TCMs.

Since only a few phytochemicals have been proven effective, there is a need for a rapid and sensitive method to screen their pharmacological activities or toxicities in TCMs. Current methods include biological chromatography column and immobilized liposome chromatography (ILC), as well as cell membrane chromatography (CMC). *In vitro*, CMC simulates the drug-receptor interaction by utilizing cell membrane receptors and silica gel carriers as the stationary phase. It is a type of bio-affinity chromatography that explores the mechanism of TCMs at the molecular level. Typically,

Table 4
Application of LC × LC in herbal analysis.

Samples	Columns		Detectors	References
	^{1}D	^{2}D		
P. notoginseng: inflorescences and leaves	XBridge amide (150 mm × 2.1 mm, 2.5 μm)	Thermo Fisher Accucore C_{18} (50 mm × 4.6 mm, 2.6 μm)	LTQ-MS	Ma et al., 2020
B. davidii: roots, stems, leaves, flowers, fruits	C_{18} (150 mm × 1.0 mm, 2.6 μm)	Cys-HILIC (150 mm × 3.0 mm, 5 μm)	UV, Q-TOF MS	Chen, Montero, Luo, Li, & Schmitz, 2020
M. cordata: roots	Symmetry Shield C_{18} (150 mm × 4.6 mm, 5 μm)	Ultimate XB C_{18} (100 mm × 4.6 mm, 5 μm)	MS	Hu et al., 2020
Safflower (Honghua): flowers	XBridge Amide (150 mm × 4.6 mm, 3.5 μm)	Ultimate amide (50 mm × 4.6 mm, 5 μm)	DAD; ESI-MS	Wang et al., 2021
P. calvata (Guangshiwei): stems, leaves	CMC column	Capcell-C_{18} (100 mm × 3.0 mm, 3 μm)	Q-TOF-MS	Pan et al., 2021
A. membranaceus (Huangqi): roots	NK-92MI/CMC (10 mm × 2 mm, 5 μm)	XBridge TM C_{18} (100 mm × 3.0 mm, 3.5 μm)	TOF-MS	Chai et al., 2022
S. baicalensis (Huangqin): roots	CMC column	XBridge TM C_{18} (100 mm × 3.0 mm, 3.5 μm)	TOF-MS	Chen et al., 2021
Ziziphus jujuba Mill. var. *spinosa* (Bunge) Hu ex H. F. Chou (Suanzaoren): seeds	PAC (2.1 mm × 150 mm, 5 μm)	BEH Shield C_{18} (3 mm × 50 mm, 1.7 μm)	Q-TOF-MS	Zhao et al., 2023b
Dendrobium species	SB C_{18} (2.1 mm × 100 mm, 1.8 μm)	Poroshell 120 Bonus (3.0 mm × 50 mm, 1.8 μm)	Q-TOF-MS	Dong et al., 2020
Alkaloids in *M. cordata*: roots	Symmetry Shield C_{18} (150 mm × 4.6 mm, 5 μm)	Ultimate XB C_{18} (100 mm × 4.6 mm, 5 μm)	MS	Hu et al., 2020
Niuhuang Shangqing Pill (NSP)	ACQUITY UPLC HSS CYANO (100 mm × 2.1 mm, 1.8 μm)	Kinetex XB-C_{18} (50 mm × 3 mm, 2.6 μm)	UV	Wu, Liang, Liang, & Xiong, 2022
Xiaoer-Feire-Kechuan oral liquid	CSH C_{18} (2.1 mm × 100 mm, 1.7 μm)	Phenyl-Hexyl (3.0 mm × 50 mm, 2.7 μm)	MS	Shang et al., 2021

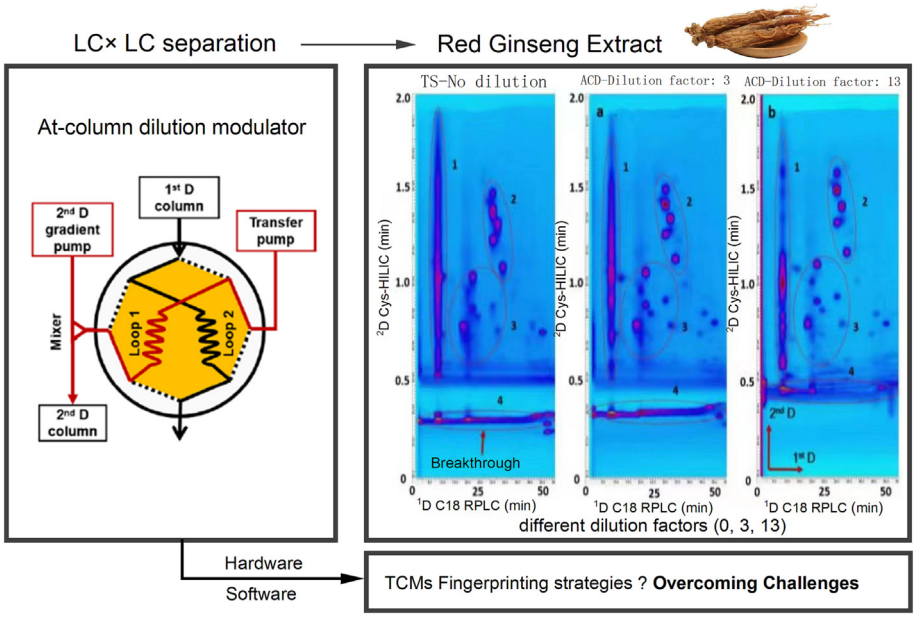

Fig. 5. Construction of an at-column dilution modulator for precise control of dilution factors to overcome mobile phase incompatibility. Application in the extracts of red ginseng with RPLC × HILIC separation, testing different dilution factors (0, 3, 13).

CMC is coupled with HPLC-MS to form an on-line 2D-LC system for the separation and screening of active components in natural products (Ping, Zhang, Wang, & Schepdael, 2022). Researchers have screened the potential anti-crystalline kidney injury (CIKI) components of *Pyrrosia calvata* (Bak.) Ching based on an on-line 2D-LC system (Pan et al., 2021). Scholars have prepared a P-glycoprotein immobilized cell membrane stationary phase (P-gp/CMSP) and integrated it into a comprehensive 2D P-gp/CMC/Capcell-C18/TOFMS system to screen five compounds in *Scutellariae Radix* (roots of *S. baicalensis*) (Liu, Wang, Gu, Zhang, & Hong, 2020). Experts have loaded a CMC column based on the construction of the hepatocellular carcinoma cell line SK-Hep1-GPC3, screening the anti-tumor components from *Scutellariae Radix* (roots of *S. baicalensis*) (Chen et al., 2021 a). Another study established a comprehensive two-dimensional CMC/C18 TOFMS system to screen potential NK cell activators in *Astragali Radix* (roots of *A. membranaceus*) (Chai et al., 2022). These studies effectively identified active components and their targets, provided effective approaches for searching potential drugs from TCMs, and offered new insights into understanding the complex action mechanisms of TCMs.

However, the LC × LC system also faces certain challenges, such as lower detection sensitivity compared to 1D-LC and mobile phase incompatibility, which limits its applicability (Pirok & Schoenmakers, 2018). Therefore, the development of hardware for LC × LC has encountered obstacles. Several methods have been developed to improve the performance of on-line LC × LC systems. For example, the use of parallel column arrays in the 2D dimension achieves complementarity between rapid sampling rates and 2D separation, maximizing peak capacity (Foster et al., 2022). In another approach, detection limits were enhanced and the dilution problem was addressed in 2D-LC by combining a broader ^{1}D column with a narrower ^{2}D column (Wicht et al., 2022). To overcome the issue of mobile phase incompatibility, researchers have proposed various strategies, including double column modulation, vacuum-assisted solvent evaporation modulation, solvent switch modulation, and on-line dilution modulation with bypass (Chapel & Heinisch, 2022). In a recent study (Fig. 5), an at-column dilution modulator was developed for flexible and precise control of dilution factors to overcome mobile phase incompatibility. This modulator, coupled with RPLC × HILIC separation, was applied to the analysis of extracts from red ginseng (roots of *P. ginseng*), resulting in the determination of more than 50 analytes under optimized conditions (dilution factor: 13). This approach exhibited sharper peaks, better separation, and increased sensitivity (Chen, Wu, et al., 2019; Chen, Li, & Schmitz, 2019). Additionally, an active-modulation strategy based on concentrating the fractions of the ^{1}D effluent was proposed (Baglai et al., 2018). The most common technology used for this purpose is stationary phase-assisted modulation (SPAM), although it has the disadvantage of premature elution of analytes (Den Uijl et al., 2022).

3.2.3. Chemometric approaches for LC × LC fingerprints

The on-line LC × LC system generates a massive amount of data, which necessitates the use of data processing tools. Various commercial software, such as GC Image LC × LC Edition, are commonly employed for visualizing, analyzing, and processing 2D-LC data. However, the abundance of herbal component information requires the application of chemometric strategies to extract valuable knowledge, particularly in the case of non-targeted scanning. To address the challenge of unresolved analytes of interest, a multivariate curve resolution-based approach has been proposed for resolving LC × LC-MS data (Pérez-Cova, Jaumot, & Tauler, 2021). Researchers have reviewed the most common chemometric tools and approaches for analyzing MDGC or MDLC data, including data compression, peak detection, peak alignment, background subtraction, and more (Navarro-Reig, Bedia, Tauler, & Jaumot, 2018). Furthermore, state-of-the-art approaches for chromatographic fingerprinting of 2D peak patterns have been thoroughly reviewed, encompassing different types of features, the development of new algorithms for 2D chromatographic misalignment, and parallel detection (Stilo et al., 2021). These fingerprint processing approaches are also applicable to LC × LC data and contribute to better control of the quality of complex TCMs. In 2022, a spectrum-effect based method was developed for screening antibacterial constituents in Niuhuang Shangqing Pill using LC × LC fingerprinting (Wu, Liang, Liang, & Xiong, 2022). As LC × LC is still a relatively young research field, further advance-

ments in hardware and the application of chemometric methods are necessary to promote its healthy development.

4. Conclusion

Compared to traditional instruments, multidimensional chromatography exhibits higher peak capacity and has proven to be a powerful analytical tool for complex TCMs samples. This paper introduced new advancements in the application of 2D-GC, 2D-LC, and other multidimensional chromatography techniques in the field of TCMs. These techniques, aided by hardware upgrades such as modulators and detectors, are widely utilized for target separation, fingerprinting, and quality evaluation of TCMs, among other applications. By combining multidimensional chromatography with chemometric algorithm (CMC), it can further facilitate the screening and separation of bioactive components in TCMs samples. However, this field also faces new challenges. On the hardware front, current 2D separation methods are still not fully mature and require improvement across various aspects. Undoubtedly, the pursuit of higher peak capacity and orthogonality drives the implementation of new 3D chromatographic technologies. It can be predicted that 3D systems hold immense potential for characterizing extremely complex samples. On the software side, multidimensional chromatography can generate third-order four-dimensional or higher-order data sets, but data processing strategies are still imperfect and lack robustness. Looking ahead, a future direction involves leveraging chemometric algorithms and computer technology to achieve intelligent instrumentalization of multidimensional chromatographic separation and standardize the fingerprinting process of TCMs samples. Through the improvement of hardware and software mentioned above, can we achieve a deeper insight into TCMs.

CRediT authorship contribution statement

Xinyue Yang: Writing – original draft. **Pingping Zeng:** Writing – original draft. **Jin Wen:** . **Chuanlin Wang:** . **Liangyuan Yao:** Project administration. **Min He:** Conceptualization, Data curation, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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