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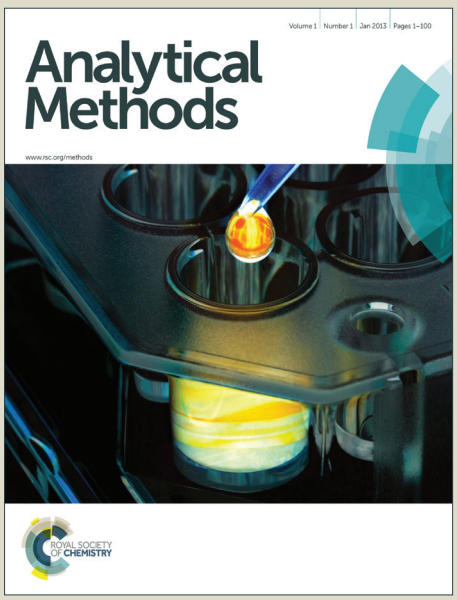

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Comprehensive two-dimensional chromatography for analyzing complex samples: recent new advances

Xianzhe Shi, Shuangyuan Wang, Qin Yang, Xin Lu, Guowang Xu*

Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

* Correspondence:

Prof. Dr. Guowang Xu, e-mail: xugw@dicp.ac.cn, Tel: 0086-411-84379530, Fax: 0086-411-84379559

Abstract

Comprehensive two dimensional chromatography (C2DC) including comprehensive two dimensional gas chromatography (GC×GC), comprehensive two dimensional liquid chromatography (LC×LC) and comprehensive two dimensional supercritical fluid chromatogram (SFC×SFC) have become effective tools to separate complex samples. GC×GC is suitable for the separation of volatile and semi-volatile compounds while LC×LC and SFC×SFC for semi- and non-volatile compounds. This review highlights the fundamental advances of C2DC on the interface techniques, orthogonality and data handling in the recent years. The applications of C2DC methods were summarized in petrochemicals, medicines, food, metabolomics, environment, etc.

Key words: Comprehensive two dimensional chromatography; GC×GC; LC×LC; Complex sample

Contents

1. Introduction

Comprehensive two-dimensional chromatography (C2DC) has been regarded as a powerful approach for complex sample analysis such as crude oil, foods, environmental and biological mixtures. The main innovation of this technique is the connection of two columns with complementary polarity of stationary phases. The fractions eluted from the first column are trapped and injected into the second one for further separation. Therefore, each component is separated twice respectively on the first and second dimensions. Thus, C2DC has much higher resolution and peak capacity in contrast to conventional one dimensional chromatography (1DC) [1-3].

C2DC mainly includes comprehensive two dimensional gas chromatography (GC×GC), comprehensive two dimensional liquid chromatography (LC×LC) and comprehensive two dimensional supercritical fluid chromatogram (SFC×SFC). GC×GC is suitable for the separation of volatile and semi-volatile compounds while LC×LC and SFC×SFC for thermally labile and non-volatile compounds. Orthogonality is very important in C2DC study, which is directly correlated with the peak capacity of C2DC. The orthogonality of GC×GC is achieved by using a column set with non-polar and polar stationary phases. To LC×LC, it consists of two different separation mechanisms based on adsorption, ion exchange, affinity and exclusion chromatography in the two dimensions as well as the mobile phase composition like pH, salt concentration etc. Therefore, the possible combinations are size exclusion chromatography × reversed-phase (RP), ion exchange × RP, normal-phase (NP) × RP, and hydrophilic interaction chromatography (HILIC) × RP and so on. In fact, the implementation of LC×LC is relatively harder than that of GC×GC because of the possible incompatibility

of mobile phases in the two dimensions of LC×LC.

Since Giddings gave the proposal of fundamental concept of two dimensional separation in 1984 [4], lots of studies on theoretical development of C2DC and its applications have been reported [5-8]. Until now, a number of reviews of C2DC have been published on experimental set-up, interfacing devices, sampling rate, peak capacity, orthogonality, data handling and applications [9-13]. Figure 1 shows the trend in publications for C2DC in the last decade. In this review we highlight the main developments and applications of C2DC for complex samples in the recent years.

Figure 1

2. Comprehensive two-dimensional gas chromatography (GC×GC)

2.1 Instrument hardware of GC×GC

The interface between the two dimensions plays a vital role in GC×GC systems. The thermal and flow modulators are overwhelmingly used to connect the two dimensional columns in GC×GC. The thermal modulator provides higher resolution and less constraint in column combinations while the flow modulation is relatively simpler to implement. Moreover, the modulation period, discharge-time and column flows can affect the performance of GC×GC. Sandra *et al.* [14] systematically evaluated the flow modulated GC×GC system for the separation of fatty acid methyl esters from bacterial cell under different column flows and modulation times. The optimal conditions were obtained using a flow ratio of about 40 between the second and first dimension separation, and a modulation time of 2 s. To manipulate and separate complex sample, Mitrevski and

Marriott developed a novel hybrid GC×GC-MDGC system, which allows the unresolved components from a slow modulation GC×GC to be transferred to a third column for further separation [7].

Column orthogonality is another most important and essential factor for GC×GC analysis. Marriott et al. developed a modeling method for orthogonality evaluation of GC×GC. The practical 2D peak distribution was characterized by using the separation properties of GC×GC without any assumptions or imposed limitations [15]. In theory, the peak capacity of GC×GC will be largest when the full orthogonality of two dimensional columns is used. Therefore, a successful GC×GC separation was achieved using the maximal polarity disparity between the primary and secondary stationary phases [9]. The current combinations of non-polar and polar stationary phases can be effective for GC×GC.

Recently, the miniaturization of GC×GC system is one of the development trends. Kim et al. designed a two-stage microfabricated thermal modulator (μTM) for two-dimensional micro-gas chromatography (μGC×μGC) by internally etched two microchannels in silicon plates. The rates of heating and cooling, power dissipation, air-gap depth and other parameters were optimized by a lumped heat transfer model. The significant advantages of μTM over the macroscale modulator included fast thermal response at a low heating power, without cryogenic fluids and negligible thermal crosstalk between two stages [16]. Furthermore, they evaluated the performance of the μTM with a set of n-alkane (C_6 - C_{10}) test. The results indicated the μTM not only had a comparable capability with the conventional modulators but also economized at least 2 orders of magnitude power [17]. To further improve the performance of μGC×μGC, Liu

et al. developed a multi-channel two-dimensional micro-gas chromatography as shown in Figure 2. The first dimensional effluent is monitored in real-time and intellectively transfers to one of the multiple second dimensional columns for further separation. Hence the separation capability of the whole μGC×μGC was greatly enhanced and finally this system was employed in analyzing workplace hazardous VOCs [18].

Figure 2

2.2 Data handling of GC×GC

The complex multidimensional data obtained from GC×GC systems need efficient processing algorithms for data acquisition and handling, peak detection and quantification, etc. [19,20]. Chemometric analysis plays a vital role in transforming these complicated data into available information [21,22]. Although the peak capacity of GC×GC is very large, overlapping of peaks is still unavoidable, especially when separating highly complicated samples. In order to resolve this issue, a new method was developed to simultaneously deconvolute and reconstruct the overlapping peak profiles in the first and second dimension of GC×GC by combining the non-linear least squares curve fitting with the exponentially modified Gaussian model (EMG). Based on this method, the profile of compounds hidden in the overlapped peaks can be simulated, and their peak areas were then determined more accurately than the previous deconvolution methods [23].

Retention indices have been proposed as an assistant tool to identify unknown compounds. Marriott *et al.* developed two methods to determine the retention time in the two dimensions of GC×GC, and the resulting retention map extended the previous

retention base range [24]. On the basis of their work, our group established the database of retention index and used it for the identification of cigarette essential oil sample, interestingly, some isomers can also be distinguished with the help of the database [25]. Zhao *et al*. built a second dimension retention map for GC×GC-TOFMS by using the large-range homologue n-alkanes from C7 to C31 as the reference compounds to make use of whole retention time space of the second dimension. As a result, the retention index values of the other compounds can be calculated directly from the linear interpolation of two consecutive n-alkanes [26]. van Stee and Brinkman developed a two-step thermodynamical algorithm to verify the secondary retention time shift of 350 compounds from a complex mixture [27]. To improve the quantitative precision of GC×GC, Synovec et al. studied the relationship among the modulation ratio (M_R), peak sampling phase and retention time variation. A good RSD of 2.1% was obtained at an average M_R of 2 and the RSD quickly increased below this M_R. The results indicated the M_R has significant implications on both quantitative precision and peak capacity production [28]. Castillo et al. developed a new software of data processing for GC×GC-TOFMS, which can afford data alignment, normalization, filtering and group-type identification, etc. Moreover, the software is suitable for rapid and efficient processing of a large number of samples, e.g. metabolomic studies [29].

2.3 Applications of GC×GC

As mentioned above, GC×GC has many advantages over conventional 1DC including better resolution, higher sensitivity, and larger peak capacity. GC×GC has been successfully applied for the separation of complex samples such as petrochemicals,

Traditional Chinese Medicines, food, metabolomics, and other complex systems.

2.3.1 Petrochemicals and Industrial products

Petroleum products contain a wide structural diversity of hydrocarbons which incorporate sulfur, nitrogen and other elements. For example, sulfur-containing compounds (SCC) existing in crude oil are quite complex mixtures consisting of thiols, sulfides, polysulfides, thiophenic and alkyl-substituted isomers of thiophenic compounds. Besides flame ionization detector (FID), some selective detectors including electron capture detector (ECD), sulfur chemiluminescence detector (SCD), nitrogenphosphorus detector (NPD), and flame photometric detector (FPD) are frequently used to increase sensitivity for the analysis of trace components. With the hyphenation of GC×GC and SCD, trace SCC were separated in crude oil fraction samples, 3620 SCC were detected in a single injection including thiophenes, benzothiophenes, dibenzothiophenes, and benzonaphthothiophenes. Compared with ASTM method, the accuracy of this method can meet the industrial requirements [30]. By using the same method, the SCC and their groups in diesel oils were investigated for quanlitative and quantitative analysis. According to the array rule of the polarity groups in the two-dimensional plane, the chromatogram can be easily divided into the four representing zones. Further, the distribution of SCC in diesel oils were found to be obviously varied from different process units, this information is helpful to improve the desulfurizated technology of diesel oil [31]. Similarly, Toussaint *et al.* used GC×GC coupled to a rapid-scanning quadrupole MS to accurately monitor the conversion of a straight run gas oil on a sulfide catalyst, it can provide a comprehensive view of transformation kinetics of this complex matrix upon catalytic conversion [32].

2.3.2 Traditional Chinese Medicine analysis

Traditional Chinese Medicines (TCMs) have great importance for clinical therapy in China and many other countries with long history. The volatile oils (VOs) of TCMs have very complex chemical constitutes, and many of them have bioactivities to treat diseases. GC×GC-TOFMS was employed to analyze the VOs of some TCMs including *Artemisia annua* L [33]. and *Pogostemon cablin Benth* [34]. As a result, much more components from *Pogostemon cablin Benth* were tentatively identified with GC×GC-TOFMS than GC-MS [34]. Moreover, the identification results of GC×GC-TOFMS are more reliable because it can provide the orthogonal information such as two-dimensional retention times and MS spectra data. Cao et al. found many active compounds of herbal medicine were lost and destructed after the sulfur-fumigated process through comparing the volatile components from sun-dried and sulfur-fumigated herbal medicine by GC×GC-TOFMS [35].

The genuineness of TCMs is one of the most crucial preconditions to guarantee quality and safety. The quality of Qianghuo of different regions was investigated based on chemical constitutes with comparison of an authentic medicinal herb collected from Sichuan province. Monoterpenes and oxygenated sesquiterpenes were found as the maker compositions to recognize geographic origin of herbs [36]. Through studying the chemical ingredient of VOs in radixes of Panax ginseng, the herb with different ages can be classified and the relative abundances of a-bisabolol, thujopsene, n-hexadecanoic acid and a-cadinol were found noticeably increasing with the age [37] .

2.3.3 Food analysis

GC×GC has been increasingly applied for food analysis such as liquor [38], wine

[39,40], tea [41] and honey [42] as well as pesticide residue analysis in food matrices [43]. Moutai is the most famous Chinese liquor, which has some unique qualities with mellow, sweet with a sauce-flavor. The volatile flavor compounds in Moutai were characterized by GC×GC-TOFMS and 528 components were identified including alcohols, ketones, organic acids, aldehydes, esters, etc. Thirty-eight organic acids clustered along three diagonals in the two-dimensional plane according to the homologous series of aromatic acids and the homologous series of fatty acids [38] As a simple and fast pretreatment method, headspace solid-phase microextraction (HS-SPME) is widely used for food analysis with simultaneous analysis by GC×GC. Dugo et al. applied this approach to monitor the volatile and semi-volatile microconstituents in Marsala wine of different ageing [44]. The similar method was also used for the analysis of volatile compounds of cacao beans [45] and the flavonoids from dark chocolate, propolis, and chrysanthemum [46], as well as the fatty acid methyl esters from algae [47]. In addition, Marriott et al. developed a high-sensitivity GC×GC-FPD method to detect the organophosphorus pesticide in six different foods including fruit, vegetable and grain. The trace pesticide residues could be found and quantitied by the valid technology [48].

2.3.4 Metabolomics

GC×GC is a suitable analytical technique for metabolomics study. More and more studies on metabolic profiling and biomarker discovery with GC×GC were reported with the increasing concerns of this research topic. Amorpha-4,11-diene synthase (ADS) is a key enzyme to catalyze artemisinin biosynthesis. Ma *et al*. applied GC×GC-TOFMS to obtain the terpenoid metabolic profilings of two lines of transgenic Artemisia annua L over-expressed and suppressed ADS, which could be separated from the control line.

Four bioprecursors of artemsisnin, monoterpenoid and diterpenoid were identified as the important metabolites in their classification [49]. GC×GC-TOFMS was not only used for metabolic profiling of known class of compounds but also used for global metabolome investigation. Li *et al*. applied a metabolomics method based on GC×GC-TOFMS to study the plasma metablites from diabetic patients and healthy controls. Five potential biomarkers including glucose and 2-hydroxybutyric acid were found, which might be useful in the diagnosis of diabetes mellitus [50]. Beckstrom *et al*. discovered some new potential biomarkers of birth asphyxia by using GC×GC-TOFMS based metabolomic analysis [51].

2.3.5 Environmental analysis

Polycyclic aromatic hydrocarbons (PAHs) and their derivatives are strong carcinogen, which present in the industrial and automobile exhaust. Fushimi *et al.* utilized thermal desorption followed by GC×GC-MS/MS to determine high-sensitively PAHs and their derivatives. Over 0.03 pg of PAHs could be detectable from trace particulate samples (10-20 μg) by this approach [52]. They further developed a GC×GC coupled with high resolution TOFMS method to detect and identify the trace organohalogens in soil, sediment and atmosphere [53]. Polychlorinated biphenyls (PCBs) in environmental samples were also separated by GC×GC-TOFMS and 200 congeners could be identified from a total of 209 PCBs [54]. Zhang et al. utilized GC×GC-TOFMS coupled with steam distillation extraction to determinate the individual nonylphenol isomers in landfill leachate and municipal wastewater. It was found that the distribution patterns of nonylphenol isomers had changed in various aquatic environments [55]. Marriott et al. applied GC×GC coupled with FID and MS for profiling analysis of phospholipid fatty

acids in forest soil samples. Some unfamiliar oxygenated fatty acid methyl esters were found and characterized by this analytical technique [56].

These typical applications with different column sets and detection methods by GC×GC are listed in Table 1.

3. Comprehensive two-dimensional Liquid chromatography (LC×LC)

3.1 Instrument hardware of LC×LC

The interface techniques of LC×LC include mainly dual loop interface, stop-flow interface and vacuum evaporation interface. The dual loop interface is mostly used in LC×LC owing to its simple structure. To eliminate the incompatibility of mobile phases used in NPLC and RPLC. Guan et al. utilized vacuum evaporation to condense the first dimensional eluents by a vacuum evaporation interface, and the second dimensional solvent redissolved the residents at the inside wall of loop for further separation in the second dimensional column [60]. However, this method has potential sample loss risk for volatile components due to evaporation in the interface. Carr et al. added a flow splitter between the two dimensions in LC×LC. Due to the full independent optimization of the two dimensions, the corrected 2D peak capacity achieved two-fold increase. Moreover, the diminished sensitivity can be partially compensated by a larger injection [61]. Stop-flow mode is applied generally when the analysis speed of the second dimension cannot match the sampling frequency of the first dimension. Mondello et al. developed a novel stop-flow LC×LC system with a sample loop for phospholipid analysis [62]. However, this stop-flow system had a drawback of long analysis time (over 6 hrs). Afterwards, our group constructed a new stop-flow interface consisting of two valves, a

make-up flow and a trap column. As a result, the dilution effect, a shortcoming of common LC×LC, was decreased, meanwhile, the sensitivity and practical peak capacity were improved [63]. Recently, selective comprehensive two-dimensional HPLC (sLC×LC) was introduced by Stoll et al. [64], which combined the advantages of both the heartcutting and comprehensive systems as shown in Figure 3.

Figure 3

Based on different separation modes, various combinations of LC×LC have been reported including SEC×RPLC, ICE×RPLC, NPLC×RPLC, HILIC×RPLC, RPLC×RPLC, HILIC×HILIC and silver ion LC×RPLC. The coupling of NPLC and RPLC is highly recommended from the point of orthogonality. However, the incompatibility of two-dimensional mobile phases is bottleneck. Dugo *et al.* used a microbore NP column at low flow rate in the first dimension and a monolithic RP column at high flow rate in the second dimension to relieve the incompatibility issue [65]. HILIC×RPLC is an alternative measure to NPLC×RPLC since it partly reduces incompatibility of two-dimensional mobile phases [66]. In addition, the recent studies indicated the incorporation of UHPLC and high temperature LC in the second dimension reduced the total analysis time, increased detection sensitivity and sampling in the first dimension [67-69]. Schoenmakers et al. developed on-line comprehensive two-dimensional ultrahigh-pressure liquid chromatography system (UHPLC×UHPLC). The column backpressure of the first and second dimensions was about 60 MPa and 85 MPa, respectively. The total analysis time was decreased to within 1 hour owing to using

a modulation time of 30 s [67]. Carr et al. developed a high-speed LC×LC system based on high temperature RPLC as the second dimension. An eluent heater was used to preheat the mobile phases of the second dimension and the column temperature was maintained at 110 °C with a column heating jacket. The cycle time of each second dimension gradient was only 21 s and the total analysis time was finished within 25 min. Meanwhile, the system had still a high peak capacity of about 900 [69].

Recently, some miniaturized systems of LC×LC have been developed. Teutenberg et al. developed a miniaturized nano-LC × capillary-LC system, which was compatible with QTOF-MS without the requirement for any flow split. This approach drastically reduced the solvent consumption compared to the conventional LC×LC, which was used successfully for the separation of a complex wastewater sample [70]. Ramsey et al. designed a hybrid multidimensional system by coupling capillary LC with a microfluidic chip. The chip integrated many functional elements including flow splitting, electroosmotic pump, capillary electrophoresis and electrospray ionization emitter. This system with TOFMS was applied to analyze a complex peptide mixture and a large peak capacity of about 1400 was acquired within the whole analytical time of 50 min [71]. Moon et al. recently developed an on-line 2DLC by coupling capillary strong anion exchange and nanoflow RPLC for comprehensive lipid analysis. A total of 303 lipids among 14 different classes were determined from human plasma [72].

3.2 Data handling of LC×LC

As GC×GC, the huge data obtained from LC×LC system also need efficient processing. Background correction is also important for peak detection and quantification

in LC×LC. In the past, much effort has been made to correct drifting baseline. Carr et al. developed a singular value decomposition-based background correction (SVD-BC) method for LC×LC with diode array detection [73] and a robust orthogonal background correction method for fast LC×LC [74], respectively. Compared with simple subtraction of a blank chromatogram and previous background correction approach of asymmetric weighted least squares (AWLS), these methods greatly reduced the background artifacts and preserved better peak intensity, and even could obtain an almost zero-mean background level.

3.3 Applications of LC×LC

3.3.1 Surfactants and industrial polymers

Surfactants usually possess different functionality type and molecular weight. Schmitz et al. applied an HILIC×RPLC method to separate simultaneously anionic, non-ionic and amphoteric surfactants. These surfactants were baseline separated by their degree of ethoxylation (EO number) in the first dimension and by their alkyl chain in the second dimension, respectively [75]. Schoenmakers et al. developed UHPLC×UHPLC to analyze the industrial polymers. The first dimension of gradient-elution UHPLC can separate the polymers based on their chemical composition, the second dimension of ultrahigh-pressure SEC allowed fast separation based on molecular size [67]. Pasch et al. constructed a novel LC×LC system by coupling solvent gradient interaction chromatography to size-exclusion chromatography (SEC). Stereoregular poly(methyl methacrylates) were separated in the two dimensions according to their taciticity and molar mass, respectively [76].

3.3.2 Medicine and food analysis

Beside volatile oils, many polar and non-volatile components exists in TCMs. Wang *et al*. built an on-line HILIC×HILIC system for the separation of hydrophilic quillaja saponaria extract [77]. Many pairs of quillaja saponin isomers were well separated and identified based on the two-dimensional retention characteristics. Dugo *et al.* utilized NPLC×RPLC to analyze carotenoids and carotenoid esters in mandarin sample and 651 of the peak capacity was obtained [78]. Beelders *et al*. applied both off-line and on-line HILIC×RPLC methods to separate the phenolic compounds in rooibos samples. The minor phenolic compounds with potential bioactivity will be detected by the approaches. The practical peak capacities can exceed 2000 and 500 for off-line and on-line modes, respectively [79]. Wang *et al.* established LC×LC system using immobilized liposome chromatography column as the first dimension and monolithic column as the second dimension. Over forty membrane permeable components were separated in a famous TCM of Schisandra chinensis. The 2D biochromatography system will favour to find strong binding bioactive components and biological fingerprint [80].

Triacylglycerides (TAGs) are a major category of complex neutral lipids that naturally exist in food. Dugo *et al*. developed a silver ion-LC×RPLC approach with ELSD detector to achieve a great number of TAGs from donkey milk fat [83] and borage officinalis oil [84]. The similar system coupled with MS detector was constructed to analyze TAGs in peanut oil and liver tissue [85]. Carotenoids is another complex compounds in food. Dugo *et al*. developed an NPLC×RPLC method under ultra high pressure conditions. Thirty-three of free carotenoids and carotenoid esters were detected and identified from a red chili peper extract with PDA and MS detection, which were

separated into ten different chemical classes in the 2D plot [86]. In addition, Villiers et al. utilized HILIC×RPLC with fluorescence detection and TOFMS to determine the tannins in the grape seed. The procyanidins with a degree of polymerization up to 16 and galloylation up to 8 could be detected and identified. [87].

3.3.3 Metabolomics and proteomics

Carr *et al*. utilized fast LC×LC with diode array detection (DAD) to separate the maize seeding digests, especially the metabolites involved with the biosynthetic pathways of indole-3-acetic acid. Several important indole acetic acid conjugates were found and identified to distinguish the mutant and wild-type maize seedings [88]. Later, they further developed a powerful approach named smart templates for peak pattern matching. The matching accuracy was improved even if the peak pattern changes under variable chromatographic conditions [89]. We applied a stop-flow HILIC×RPLC method with TOFMS for plasma lipid analysis. 372 lipids including 13 different classes were identified in positive mode and this method could be used in quantitative analysis with good linearity and repeatability [63].

LC×LC has been widely applied for the analysis of proteomics in the past years [90]. Levin et al. used an off-line SCX×RPLC coupled with TOFMS approach to profiling analysis of rat hippocampal proteome. 1340 unique proteins were identified including phosphorylated proteins and membrance receptor proteins and they were related with synaptic function. [91]. To avoid the high level of non-volatile salts in the mobile phase, Griffiths et al. utilized the porous graphitic carbon (PGC) column as the first dimension, which had good orthogonality to a traditional RP second dimension at low pH. Compared with the classical SCX-RP system, the new PGC-RP system presented better peptide

separation in a complex cell lysate digest sample [92]. Recently, on-line RPLC×HILIC system was applied to the separation of peptides from a tryptic digest of three proteins. Higher peak coverage was obtained whereas peak capacity was lower compared to RPLC×RPLC [93].

In Table 2, many typical LC×LC applications are listed with different column sets and detection methods.

4. Other types of comprehensive two-dimensional chromatography

Supercritical fluid chromatography (SFC) has unique advantages including rapid separation speed and compatibility with flame ionization detector compared with LC due to using neat carbon dioxide as the mobile phase. In addition, the large number of stationary phases developed for LC can also be used in SFC, which can provide two different separations in comprehensive two-dimensional supercritical fluid chromatography (SFC×SFC). The interface of SFC×SFC is usually similar to that of LC×LC except for extra restrictors to keep pressure stable. Therefore, SFC×SFC could be applied in various fields as a complementary technique to GC×GC and LC×LC. SFC× SFC presented similar separation capabilities on the analysis of petroleum samples heavier than middle distillates as those of GC×GC method [98]. Zeng et al developed a new SFC×SFC system coupled with MS, which integrated achiral column and chiral separations into a single run. The enantiomeric analysis of a racemic pharmaceutical compound from complex mixtures was performed without prior clean up [99]. Hirata and Ozaki developed a capillary SFC×SFC in stop-flow mode with synchronized pressure programming. In the system, different polar GC columns like DB-1, DB-17 and

DB-WAX were connected to obtain wide selectivity. The separation characteristics of this system could be easily adjusted by changing the programmed pressure rate [100].

5. Conclusions and perspectives

As a promising and powerful approach to study complex samples, C2DC was developed rapidly during the last decade. At present, high-dimensional data processing is one of the biggest challenges and bottlenecks to the wide applications of this technique. The software platforms containing statistical analysis and data visualization tools should be improved to deal with comprehensive comparison of peaks among different samples, especially for the huge 2D data in omics studies [101]. For LC×LC, to make the buffers on the first and second dimensions compatible, the fractions on the second dimension are usually greatly diluted, therefore, how to improve the detection sensitivity on the second dimension to enhance the determination of trace components in the real samples is an issue to be addressed. For GC×GC, the technique innovation has been very weak in at least last years except for the increasing reliability and improved data handling. In the future, miniaturization of GC×GC and LC×LC systems will become more common and practical, for example, in the form of two dimensional micro-GC or chips.

We believe that continuous progresses will be made on C2DC study with the new development of instrumental connection configurations, higher-efficiency columns, intelligent data processing strategies, and others.

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563 Table 1 Typical applications of GC×GC with a variety of column combinations

Samples	Column sets	interface	Detector	Ref.
Bacterial fatty acids	HP-5ms (Agilent)× BP-70 (Agilent)	Flow modulator	FID and MS	[14]
Sulfur-containing compounds in crude oils or Diesel oil	VB-5 (Valco) × 007-17 (Quadrex)	thermal modulator	SCD	[30] [31]
Conversion of a Straight run gas oil	DB-5ms (J&W) × VH-17ms (Varian)	thermal modulator	qMS	[32]
Volatile oil of *Artemisia annua* L	DB-Petro (J&W) × DB-17ht (J&W)	thermal modulator	TOFMS	[33]
Volatile oil of *Pogostemon cablin Benth*	SolGelWax (SGE) × Cyclodex-B (Agilent)	thermal modulator	TOFMS	[34]
Volatile components from sun-dried and sulfur-fumigated herbal medicine	DB-5ms (J&W) DB-17ht (J&W)	thermal modulator	TOFMS	[35]
Volatile oil in the rhizomes and radixes of *Notopterygium incisum Ting*	CEC-1 (Chrom Expert Company) × DB-WAX (J&W)	thermal modulator	TOFMS FID	[36]
Volatile oil in the radixes of Panax ginseng	DB-5ms (J&W) × DB-1701(J&W)	thermal modulator	TOFMS FID	[37]
Flavor compounds in Chinese Moutai liquor	HP-Innowax (Agilent) × DB-1701(J&W) or DB-Petro (J&W) × DB-1701 (J&W	thermal modulator	TOFMS FID	[38]
Volatile components of wine	VF-5ms (Varian)× VF-17ms (Varian)	thermal modulator	TOFMS	[39]

Volatile profile of Brazilian Merlot wines	DB-5 (J&W)× DB-wax (J&W) DB-wax (J&W)× DB-1ms (J&W) DB-wax (J&W) × DB-17ms (J&W)	thermal modulator	TOFMS	[40]
Volatile components in green, oolong and black teas	DB-5ms (J&W) × Rtx-200ms (Restek)	thermal modulator	TOFMS	[41]
Artifacts in honey volatiles	HP-5 (Agilent) × Wax-10 M (Supelco)	thermal modulator	TOFMS FID	[42]
Volatile composition of Marsala wines	SLB-5 ms (Supelco) × Wax-10 (Supelco)	thermal modulator	MS, FID	[44]
Volatile compounds of cacao beans	Rtx-5ms (Restek) × Rtx-200ms (Restek)	thermal modulator	TOFMS	[45]
Flavonoids in dark chocolate, propolis, and chrysanthemum	BPX5 (SGE) × BPX50 (SGE)	thermal modulator	TOFMS FID	[46]
Fatty acids in marine biota	DB-1ms (J&W) × SLB-IL 82 and SLB-IL 100 (Supelco)	Flow modulator	FID	[47]
Terpenoid metabolic profiling analysis of Transgenic Artemisia annua L	DB-wax (J&W)× DB-1701 (J&W)	thermal modulator	TOFMS	[49]
Biomarker discovery for diabetes mellitus	DB-5 (J&W) × DB-1701 (J&W)	thermal modulator	TOFMS	[50]
Potential biomarkers of perinatal asphyxia in a non-human primate	Rtx-5ms (Restek)× Rtx-200ms (Restek)	thermal modulator	TOFMS	[51]

model				
Polycyclic aromatic hydrocarbons and their derivatives	InertCap-5ms / Sil (GL) × BPX-50(SGE)	thermal modulator	MS/MS	[52]
Organohalogens in soil, sediment and the atmosphere etc	InertCap 5ms/Sil (GL) × BPX-50 (SGE)	thermal modulator	TOFMS	[53]
Polychlorinated biphenyls in environmental samples	Rtx-PCB (Restek)× Rxi-17 (Restek)	thermal modulator	TOFMS	[54]
Nonylphenol isomers in landfill leachate and municipal wastewater	DB-5ms (J&W) ×Wax-10 (Supelco)	two-stage quad jet thermal modulator	TOFMS	[55]
Phospholipid fatty acids in forest soil samples	HP-5ms (Agilent) × HP-Wax (Agilent)	thermal modulator	FID TOFMS	[56]
Essential oils in rosemary and oregano	HP-5ms (J&W) × DB-17ms (J&W)	thermal modulator	FID / MS	[57]
Toxaphene congeners in soil	DB-XLB (J&W) × BPX50 (SGE)	thermal modulator	MS	[58]
Non-sreroidal anti-inflammatoru drug residues in wastewater and surface water	xi-5Sil MS × BPX-50 (SGE)	thermal modulator	TOFMS	[59]

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565 Table 2 LC×LC separation with a variety of column combinations and their applications

Samples	Column sets	Interface	Detection	Ref.
Phospholipids from cow's milk and plasma	Ascentis express HILIC (Supelco) ×Ascentis express C18 (Supelco)	a 10-port valve with two 100 μL loop (stop-flow mode)	MS	[62]
Lipids from human plasma	Acquity BEH HILIC (Waters) ×Acquity BEH C8 (Waters)	8-port and 10-port valves with a trap column and make-up flow (stop-flow mode)	TOFMS	[63]
Oxygen heterocyclic fraction in lemon oil	Sil LC-SI column (Supelco) ×Chromolith Flash (Merck)	a ten-port valve with two 20 μL loop	PDA	[65]
di- to deca-oligonucleotides	Ascentis Silica (Supelco) × XBridge C18 (Waters)	a ten-port valve with two ODS cartridges	UV, ESI/LCQ ion trap	[66]
Industrial polymers	Acquity BEH C18 (Waters) × Acquity BEH HILIC (Waters)	a ten-port valve with two 100 μL loop	PDA and ELSD	[67]
Wastewater sample	Hypercarb column (Thermo) × SunShell C18 (homemade)	a ten-port valve with two 1.57 μL loop	TripleTOF	[70]

Anionic, non-ionic and amphoteric surfactants.	ZIC-HILIC column (Merck) × Reprosphere 100 C8-Aqua column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany)	a ten-port valve with two 25 or 50 µL loop	QTOF MS	[75]
Stereoregular poly(methyl methacrylates)	Hypercarb column (Thermo) × PL gel Mixed E column (Varian)	a 8-port valve with two 100 µL loop	evaporative light scattering detector	[76]
Saponins in Quillaja saponaria	TSKgel Amide-80 (Tosoh) ×PolyHydroxyethyl A (homemade)	a ten-port valve with two 100 µL loop	ESI/Q-TOF-MS	[77]
Carotenoids and carotenoid esters in mandarin essential oil	Supelcosil LC-Si (Supelco) × Chromolith RP-18 (Merck)	a ten-port valve with two 50 µL loop	PDA, APCI MS	[78]
Phenolic compounds in rooibos samples	Develosil Diol-100 (Nomura Chemical) ×Zorbax SB-C18 (Agilent)	a ten-port valve with two 5 µL loop	PDA	[79]
Schisandra chinensis	immobilized liposome chromatography column (homemade) × monolithic column (homemade)	a ten-port valve with two 500 µL loop	PDA, MS	[80]
Phenolic compounds from apple	Lichrospher diol-5 (HiChrom) ×Ascentis Express C18 (Supelco)	a ten-port valve with two 27.3 µL loop	UV and MS	[81]
Pesticides in various	YMC-Pack Diol (YMC) ×	6 and 10-port	MS/MS	[82]

foods	Poroshell 120EC-C18 (Agilent)	valves with a packed loop of Zorbax SB-C8		
TAGs in donkey milk fat	Ag^+ column (Homemade) × Chromolith RP-18 (Merck)	a ten-port valve with two 20 μL loop	ELSD APCI MS	[83]
TAGs in borage officinalis oil	Ag^+ column (Homemade) × Ascentis Express C18 (Supelco)	a ten-port valve with two 11 μL loop	ELSD	[84]
TAGs in peanut oil and mouse liver	Ag^+ column (Homemade) × EPS C18 (Bischoff)	a ten-port valve with two 20 μL loop	APCI MS	[85]
Carotenoid in red chili peppers	Ascentis ES Cyano (Supelco) × Ascentis Express C18 (Supelco)	two six-port valves with 10 μL loop	PDA, APCI MS	[86]
Tannins in Grape Seed	Develosil Diol-100 (Nomura Chemical Co.) × Zorbax SB-C18 (Agilent)	a ten-port valve with two 5 μL loop	QTOF-MS	[87]
Metabolites from maize seeding digests	Discovery HS-F5 (Sigma-Aldrich) × prototype carbon-clad zirconia reversed-phase (Homemade)	a ten-port valve with two 34 μL loop	UV	[88]
Rat hippocampal proteome	Polysulfoethyl ATM SCX (PolyLC) × BEH C18 (Waters)	off-line mode	QTOF-MS	[91]
Peptide in a cell lysate	PGC (Thermo) × BEH C18	off-line mode	LTQ Orbitrap	[92]

digest sample	(Waters)		MS	
Phenytoin in urban wastewater	Ascentis Express F5 (Supelco) × carbon-modified silica column (United Science)	ten-flow path selector valves connected by six 70 μL loops	PDA	[94]
Procyanidins in grape seed	Syncronis HILIC (Thermo) × Ascentis Express C18 (Supelco)	a ten-port valve with two 20 μL loop	PDA MS/MS	[95]
Furanocoumarins in apiaceous vegetables	Ascentis Express F5 (Supelco) × Ascentis Express C18 (Supelco)	ten-flow path selector valves connected by ten sample loops	PDA	[96]
Polypropylene copolymers	PLgel Olexis SEC (Agilent) × PL Rapide H (Polymer Laboratories)	a 8-port valve with two 100 μL loop	infrared spectroscopy	[97]

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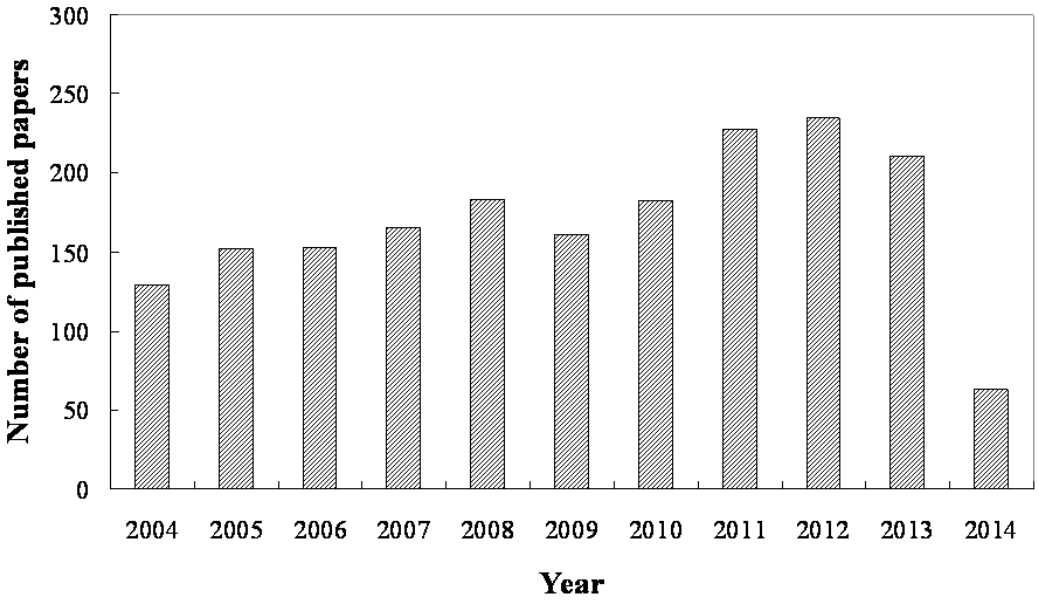
Legends

Figure 1 Number of publications of comprehensive two dimensional chromatography in the last decade (data up to June 2014). The sources of database are the Science Citation Index Expanded from the Web of Science. The search keywords are (comprehensive two dimensional chromatography) OR (comprehensive two dimensional gas chromatography) OR (comprehensive two dimensional liquid chromatography) OR (comprehensive two-dimensional Supercritical fluid chromatography) OR (GC×GC) OR (LC×LC) OR (SFC×SFC).

Figure 2 Schematic representation of the proposed smart μGC×μGC with dual 2nd columns. Reproduced with permission from Ref. [28] Copyright (2013) Royal Society of Chemistry

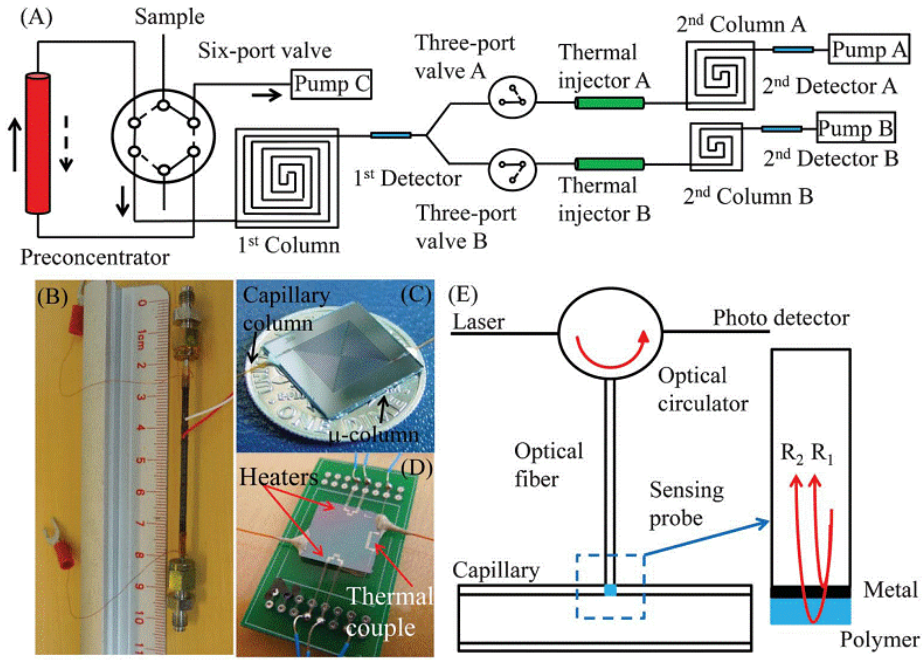
Figure 3 Schematic representation of instrument configuration for sLC×LC. Reproduced with permission from [19] Copyright (2012) Elsevier



585

586 Figure 1

587



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589 Figure 2

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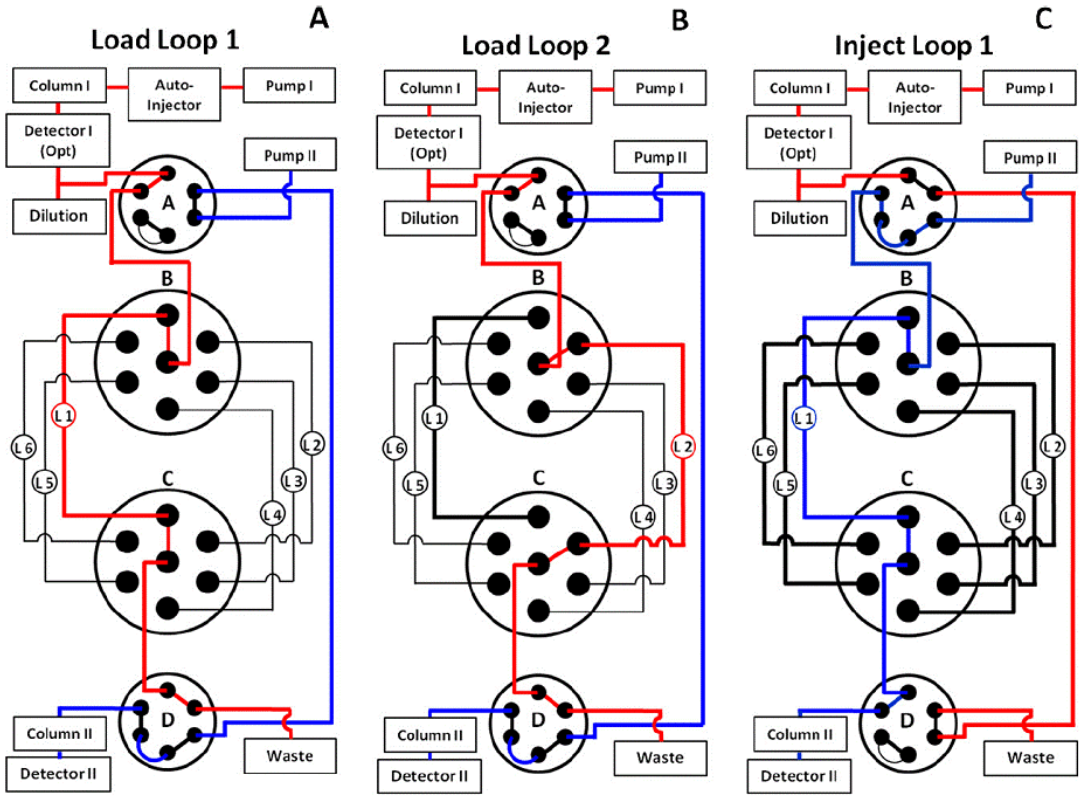

Figure 3