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Review

Recent developments and contributions from Chinese scientists in multidimensional separations for proteomics and traditional Chinese medicines

The most basic task in proteomics remains the detection and identification of proteins from a biological sample, and the most traditional way to achieve this goal consists in protein separations performed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Yet the 2-D PAGE-mass spectrometry (MS) approach has its drawbacks with regard to automation, sensitivity, and throughput. Consequently, considerable effort has been devoted to the development of non-gel-based proteome separation technologies in an effort to alleviate the shortcomings of 2-D PAGE. In addition, traditional Chinese medicines (TCMs), due to their long period of clinical testing and reliable therapeutic efficacy, are attracting increased global attention. However, hundreds or even thousands of components are usually present in TCMs, which results in great difficulties of separation. As a mainstream separation tool, multidimensional liquid separation systems have shown powerful separation ability, high peak capacity, and excellent detectability in the analysis of complex samples including biological samples and TCMs, *etc.* Therefore, this review emphasizes the most recent advances in multidimensional liquid chromatography and capillary electrophoresis-based separation techniques, and the corresponding applications in proteomics and TCMs. In view of the significant contributions from Chinese scientists, this review focuses mainly on the work of Chinese scientists in the above fields.

Keywords: Electrophoresis / Multidimensional chromatography / Proteomics / Separation / Traditional Chinese medicines

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1 Introduction

Since the 1950's, scientific interest has been increasingly shifting to molecular biology leading to the accomplishment of gene sequencing in the Human Genome Project (HGP). Proteomics has been the focus due to the role of executor of the life function. Proteomics refers to the analysis of all the proteins expressed in a cell or tissue. 2-D PAGE is the most universal method for proteomics and possesses high resolving power for proteins. Some problems in 2-D PAGE, however, are still difficult to overcome, such as limited pH range, time-consumption, and inefficient sensitivity for low abundance proteins. This reality has unleashed considerable effort in developing new proteomics techniques as alternatives to 2-D PAGE. In contrast to 2-D PAGE, multi-dimensional liquid chromatography has emerged with obvious advantages of availability of various chromatographic modes offering different separation mechanisms, automation, high resolution, sensitivity, reproducibility, and high throughput. Multidimensional separation techniques, including liquid chromatography-liquid chromatography (LC-LC) [1 – 3], capillary electrophoresis-capillary electrophoresis (CE-CE) [4, 5], liquid chromatography-capillary electrophoresis (LC-CE) [6, 7], *etc.*, have attracted much attention in recent years. They have been widely used for separation of biological samples [8, 9], polymers [10], TCMs [11, 12], and other complex mixtures [13].

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Abbreviations: CNGSE, capillary non-gel sieving electrophoresis; **CSE,** capillary sieving electrophoresis; **HGP,** Human Genome Project; **IMAC,** immobilized metal affinity chromatography; **FITC,** fluorescein isothiocyanate; **LIEF,** liquid isoelectric focusing; **MulPIT,** multidimensional proteins identification technology; **SCX,** strong cation exchange chromatography; **SEC,** size exclusion chromatography; **TCMs,** traditional Chinese medicines; **WCID,** whole column imaging detection

TCM as we know it today has a long history dating back several thousands of years. With the development of theory and clinical practice, China has accumulated a rich body of empirical knowledge about the use of medicinal plants for the treatment of various diseases. So far, there have been 12 806 medical resources found in China, including 11 145 medicinal plants, 1581 medicinal animals, and 80 medicinal minerals [14]. Furthermore, a total of 2375 products have been compiled in the Pharmacopoeia of the People's Republic of China (2000 edition) [15]. Such ample Chinese natural medicinal resources provide valuable materials for the discovery and development of new drugs of natural origin. Separation and analysis of the components in TCMs is an important subject in order to ensure the reliability and repeatability of pharmacological and clinical research. In past decades, a large number of effective analytical tools have been used for analyzing constituents of TCMs, especially multidimensional chromatographic methods, and provide an impressive range of separation techniques for complex mixtures.

This review will summarize the state of the art of multidimensional liquid chromatography and electrophoresis in China, and multidimensional chromatography methods for separation and analysis of complex compounds in proteomics and TCMs will receive special emphasis.

2 Multidimensional separation modes for proteomics

2.1 LC-LC

The advent of proteomics research also imparted a great boost to the development of the related technologies of multidimensional liquid chromatography. Many research groups have worked extensively in this field in China. An offline system, coupling SCX with RPLC, was developed for the analysis of proteins extracted from the cancer tissue of nude mice inoculated by a human hepatocellular carcinoma cell line D20 by Yang *et al.* [16]. An online 2-D separation system [17–18], coupling SCX with cRPLC, was developed further to separate proteins associated with hepatocellular carcinoma. By using this system, complex samples could be injected, desalted, separated, and analyzed in completely automated manner. The procedure for proteomics analysis was applied for proteins with a very high molecular mass (>100 000), small molecular mass (<20 000), of high basicity (p*I* > 9.5) and hydrophobicity, which are not well resolved in two-dimensional gel electrophoresis (2-DE). In total 229 proteins were identified by using the described proteomics platform. Zhang *et al.* further combined 2-D LC separation with a parallel MS/MS identification for proteome analysis of complicated samples [19]. The identification results demonstrated that the complementary nature of MALDI-based and ESI-based analysis could improve proteome coverage and protein identification confidence, especially for those one peptide-matching proteins identified in the single MS/MS analysis route. Zou *et al.* developed a strategy for human plasma proteome research: prefractionation of human plasma proteins using online strong cation exchange chromatography/reverse phase liquid chromatography (SCX/RPLC) followed by trypsin digestion and capillary RPLC-ESI-Linear Ion Trap-MS/MS identification, followed by bioinformatics annotation, which provided as a high-throughput, sensitive, and effective analytical approach for proteomics of human plasma [20]. A total of 1292 distinct proteins were successfully identified, among which some proteins known to be present in serum at <10 ng/mL were detected. A comprehensive 2-D liquid-phase separation system, coupling SCX to RPLC, instead of specificity depletion method, was developed at the intact protein level for depletion of high abundance proteins from rat liver [21]. A total of 77 high abundance proteins were depleted in one experimental run. The ratio of depleted content of high abundance proteins to that of total proteins was about 34.5%. In total 1530 proteins were identified using the depletion strategy. Qian and co-workers constructed a robust and automatic immobilized metal affinity chromatography (IMAC)-capillary RPLC-ESI MS/MS technology platform, by which all procedures needed in phosphopeptide analysis including IMAC enrichment, RPLC separation, and nanospray MS/MS can be done automatically under the control of the MassLynx program [22]. The platform was applied to the identification of phosphorylation sites of recombinant human telomeric repeat binding factor 1 treated with kinase *in vitro*, and two phosphorylation sites are defined. With the development of technology, multidimensional liquid chromatography techniques have been used widely due to complete automation, high resolution, low-cost, and high sensitivity, *etc*. Multidimensional proteins identification technology (MulPIT) has been applied to the analysis of mouse liver plasma membrane proteins [23], mitochondria proteome of human fetal liver [24], subcellular fractions from rat liver (crude mitochondria, cytosol, purified mitochondrial fraction, total liver) [25], and SARS associated coronavirus [26], *etc.* MulPIT was also modified further by using an integrated column, containing both SCX and RPLC sections for 2-D LC. The peptide mixture was fractionated by a pH step using a series of pH buffers, followed by RPLC [27]. Since no salt was used during separation, the integrated multidimensional LC can be directly connected to mass spectrometry for peptide analysis. More than 2000 proteins in mouse liver were identified by this method. The peptides were eluted according to their p*I* distribution. The resolution of the pH fractionation is about 0.5 pH units. In recent years,

there has been increased effort focused on the separation, quantification, and identification of all proteins in a cell or tissue. The difficulty of analyzing the proteome is that many low-abundance proteins are not detected due to a predicted dynamic range of up to five orders of magnitude [28]. Therefore, the field of proteomics requires the development of effective fractionation and separation methods that offer high sensitivity and wide dynamic range to detect more proteins in a cell or tissue [29]. Liquid isoelectric focusing (LIEF) as the prefractionation technology was used to crudely separate sample prior to 2-D-LC-MS/MS [30]. A LIEF-2-D-LC-MS/MS system was applied to the proteome of *Saccharomyces cerevisiae*. An approach for fractionating complex protein samples from rat liver prior to 2-DE using RPLC was described [31]. The whole lysate of liver tissue was prefractionated by RPLC. Successive fractions were analyzed using 2-DE and identified by MALDI-TOF-TOF mass spectrometry. Qian and co-workers have compared the results from five different techniques using the same HUPO Plasma Proteome Project (PPP) reference serum specimen with the six proteins of highest abundance depleted by affinity chromatography [32]. The approaches compared were (i) intact protein fractionation by anion exchange chromatography (WAX) followed by 2-DE and MALDI-TOF-MS/MS for protein identification (2-DE strategy); (ii) intact protein fractionation by 2-D HPLC and then coupled with solution digestion of each fraction and microcapillary RPLC microESI-MS/MS identification (protein prefractionation strategy); (iii) digestion of mixed proteins by trypsin followed by automated online microcapillary 2-D HPLC with IT microESI-MS/MS (online shotgun strategy); (iv) same as (iii) with the strong cation exchange chromatography (SCX) step performed offline (offline shotgun strategy); and (v) same as (iv) with the SCX fractions reanalyzed by optimized nanoRPLC-nanoESI-MS/MS (offline shotgun-nanospray strategy). All five approaches yielded complementary sets of protein identifications. The total number of unique proteins identified by each of these five approaches was (i) 78, (ii) 179, (iii) 131, (iv) 224, and (v) 330, respectively. In all, 560 unique proteins were identified. One hundred and sixty-five proteins were identified through two or more peptides. Only 37 proteins were identified by all five approaches.

2.2 CE-CE

2-D CE has been a powerful tool for the separation of proteins and peptides in proteomics, due to its minimal sample consumption, high separation efficiency, high separation speed, low-cost, and compatibility with biological materials.

Dovichi and co-workers reported fully automated 2-D micellar electrokinetic capillary chromatography (MEKC) for protein analysis [33]. In the system, proteins

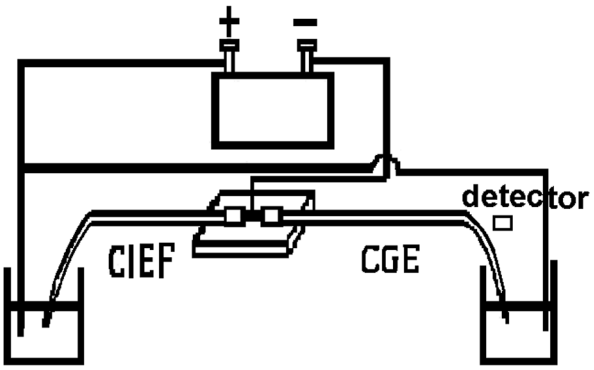

Figure 1. Construction of 2-D CIEF-CGE framework. Reprinted from [38] with permission.

are labeled with the fluorogenic reagent 3-(2-furoyl)-quinoline-2-carboxaldehyde. Laser-induced fluorescence was used as an ultrasensitive detector and detection limits of a few zeptomoles of labeled protein were achieved. A similar 2-D electrophoresis instrument [34] was used to couple capillary sieving electrophoresis (CSE) with micellar electrokinetic capillary chromatography (MEKC). This technology was applied to separate the fluorescently labeled bacterium *Deinococcus radiodurans* protein homogenate. Over 150 components were resolved from the homogenate. The protein expression in single mammalian cells was also studied by using CSE-MEKC technology [35]. The value of this technology was demonstrated by the generation of protein fingerprints from single native MC3T3-E1 osteoprogenitor cells, MC3T3-E1 cells transfected with the human transcription regulator TWIST and single-cell protein fingerprints from MCF-7 breast cancer cells before and following treatment to induce apoptosis. In Mohan's group [36], a 2-D separation system, CIEF on-line coupling with transient isotachophoresis-zone electrophoresis (CITP-CZE), was constructed. The maximum peak capacity is estimated to be around 1600. Pawliszyn and co-workers developed a 2-D system, coupling MEKC to CIEF, by using a 10-port valve with two conditioning loop device [37]. Whole-column-imaging detection was investigated for application in this system.

Zhang and co-workers combined CIEF with capillary gel eletrophoresis (CGE) [38], by mounting a hollow fiber on a methacrylate resin plate (Fig. 1). The two dimensions of capillary shared a cathode fixed in a reservoir in the methacrylate plate; the system thus had three electrodes and only one high-voltage source. With the dialysis interface, small molecules like sodium dodecyl sulfate (SDS) can be mixed with analytes prior to CGE separation. Chemical mobilization was utilized to drive the sample zones of the first dimension into the second dimension capillary for further separation. CIEF coupling of the capillary zone electrophoresis (CZE) system was also developed using a similar dialysis instrument [39]. The

focused zone in the first dimension was driven to the dialysis interface by electroosmotic flow (EOF), besides chemical mobilization from the first anode to the shared cathode. And then, in the second dimension, the focused zone was further separated and driven by an inverted EOF, which originated from the charged layer of a cationic surfactant adsorbed onto the inner wall of the capillary. Zhang's group also developed a 2-D system, by coupling CIEF with capillary non-gel sieving electrophoresis (CNGSE) for protein separation [40, 41]. In principle, this 2-D prototype system is similar to the classic 2-D PAGE. The feasibility of a complete on-line combination of CIEF and CNGSE for protein analysis has been demonstrated by examining each technique independently for the separation of hemoglobin and protein mixtures excreting from lung cancer cells of rat. An on-column etched fused-silica porous junction was designed for on-line coupling of CIEF with CZE [42]. The two separation columns were integrated on a single piece of fused-silica capillary through an etched 4- to 5-mm length porous junction along the capillary. The primary advantages of this novel porous junction interface over microdialysis hollow fiber membrane are zero dead volume, simplicity, and ruggedness, which make it particularly well suited for an online coupled capillary electrophoresis-based multiple dimensional separation system. The performance of the 2-D CIEF-CZE system constructed with such an etched porous junction was evaluated by analysis of protein mixtures.

Capillary electrophoresis is especially suitable as the second dimension separation stage in a multidimensional system, due to its high separation rate, high separation efficiency, and minimal sample consumption. RPLC with CZE is the most common 2-D system because of its many advantages. RPLC and CZE are presumably highly orthogonal separation methods. RPLC separates analytes on the basis of hydrophobicity and CZE separates analytes mainly on the basis of charge, and to a lesser degree by size. On this basis, they are good candidates for pairing in a 2-D system. In addition, the buffer of the two modes is compatible.

For the first time, Jorgenson and co-workers [43] developed an automated coupling RPLC and CZE system using a computer-controlled six-port valve-loop interface. Effluent from the RP column filled a loop. A second pump flushes this loop material over the grounded (anode) end of a CZE capillary at specific intervals. Electromigration injections are performed on the CZE portion of the system from a flowing stream. Fluorescence detection is used on the CZE capillary. The system is used for the analysis of peptide standards and fluorescently labeled peptide products from a tryptic digest of ovalbumin. In 1993, a comprehensive 2-D system was constructed by coupling size exclusion chromatography (SEC) with CZE [44]. The union between the two capillaries was made through a coaxial sleeve. The outlet of the SEC micro-column was positioned directly across from the inlet of the electrophoresis capillary, separated only 127 μm. The flow of CZE buffer carried SEC effluent away to waste, preventing transfer of sample to the CZE capillary. When an injection was desired, this flush flow was interrupted, allowing the flow of SEC effluent to carry sample into the narrow gap separating the SEC micro-column and the CZE capillary. This coupling was named a transverse flow-gated interface. The system was modified and improved [45] by using a clear polycarbonate polymer, Lexan. The interface was transparent and allowed direct observation of the transfer of sample from LC to CZE. A 3-D system was further developed by coupling the 2-D system of RPLC-CZE with MS [46]. Peptide standard and tryptic digests of ribonuclease B were separated and detected on-line by electrospray mass spectrometry in about 15 min. In 1991, Jorgenson and co-workers designed a "fast-CZE" instrument, by using an optical-gating injection system [47]. Optical-gating injection is based on photodecomposition of FITC induced by an argon ion laser beam. In 1995, a rapid comprehensive 2-D system was developed by coupling RPLC with the optically-gated CE instrument [48]. With the development of such a rapid 2-D analysis, a 3-D separation system was also set up using SEC and RPLC-optically-gated CE [49]. The overall peak capacity of the system was then greatly increased. Issaq *et al.* [50] used a simple off-line RPLC-CZE system to separate a mixture of protein digests, whereby fractions from RPLC were collected automatically every 30 s, concentrated under vacuum, and analyzed simultaneously by 96-array CE with laser-induced fluorescence (LIF) detection. Stroink *et al.* [51] developed a 2-D system of on-line coupling of SEC and CZE *via* a reversed-phase C18 trapping column for the analysis of structurally related enkephalins in cerebrospinal fluid.

With the advent of proteomics research, the related technologies of multidimensional liquid chromatography have also undergone considerable development. A comprehensive 2-D capillary LC and CZE system coupled with tandem mass spectrometry has been presented by Zhang and co-workers [52] (Fig. 2). This system was successfully used to separate and analyze proteins in D20 (human hepatocellular carcinoma model in nude mice with high metastatic potential) liver cancer tissue. More than 300 proteins were identified. Zhang and co-workers further fabricated the second dimension CE into chip. A novel comprehensive 2-D separation system coupling capillary reverse phase liquid chromatography (cRPLC) with microchip electrophoresis [53] was demonstrated. A valve-free gating interface was devised simply by inserting the outlet end of the LC column into the cross-channel on a specially designed chip. A laser-induced fluorescence detector was used to perform on-chip high sensitivity detection. The effectiveness of this system was demonstrated by separating peptides of the FITC-labeled tryptic

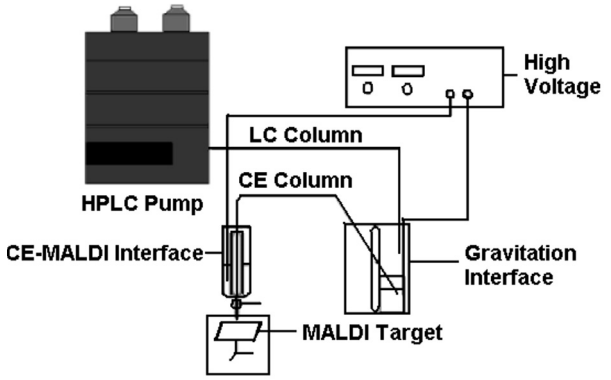

Figure 2. Schematic configuration of the comprehensive cRPLC-CE-MS system. Reprinted from [52] with permission.

digests of bovine serum albumin. The focusing effect of CIEF not only contributes to high resolution, but also may permit low-abundance analysis with a typical concentration factor of 50 – 100 times. Therefore, CIEF technology has been used widely, especially in proteomics research. Zhang and co-workers [54] demonstrated an off-line 2-D system, in which cRPLC acts as the first dimension and CIEF as the second dimension. A peak capacity of more than 10 000 has been achieved. A laser-induced fluorescence detector was used for the BSA digest. FITC and BODIPY maleimide were used to tag the proteins. This system was further modified by using a capillary array-based 2-D system [55]. An array of up to 60 capillaries was assembled as the second separation dimension. A whole column imaging detection (WCID) with LIF was developed. The efficiency of this cRPLC-array CIEF-LIF-WCID system was demonstrated using samples of soluble proteins extracted from liver cancer tissue. The overall peak capacity was estimated to be around 18 000 in an analysis time of less than 3 h. The reproducibility of consecutive runs and different columns was assessed as having an RSD of 1.5 and 2.2%, respectively, in focusing positions. HPLC coupled with MEKC was developed for the analysis of carbohydrates in Aloe powder and food [56]. The method for the derivatization of carbohydrates with 1-phenyl-3-methyl-5-pyrazolone was simplified. Good reproducibility could be obtained with RSD values of the migration times within 5.0 and 2.3%, respectively. These methods are quite useful for routine analysis of monosaccharides and oligosaccharides in real samples.

3 Multidimensional separation modes for traditional Chinese medicine

Because TCMs provide almost infinite resources for drug development, they are attracting more and more attention in modern pharmaceutical institutions. However, the compositions of TCMs are very complicated and they usually contain hundreds of chemical constituents, only a few of which may have pharmaceutical and/or toxic activity. Therefore, efficient and selective methods are required for the analysis and identification of these ingredients from TCMs.

Different analytical techniques including thin-layer chromatography (TLC) [57], gas chromatography (GC) [58], LC [59], biochromatography [60, 61], and MEKC [62] have been applied for this purpose. However, with respect to multidimensional techniques, up to now there have been only a few reports on the application of multi-dimensional separation systems coupled with mass spectrometry for the analysis of TCMs.

MEKC has proved very useful for separating different types of samples, especially for separating neutral component. A 2-D system [63], combining LC with MEKC, was designed by using a dynamic interface with pulse contact. A gas-driven device was able to drive the LC column back and forth. LC effluent was stacked in a chamber and was ejected into the cross channel when the LC column moved forward. The system was further modified and optimized [64]. In order to increase injection efficiency and diminish the injection time and sample dilution ratio during the effluent transfer from LC to CE, a high voltage injection approach was explored. The high efficiency of this system was demonstrated by separating neutral components in liquorice, a typical ingredient in TCMs. Hundreds of components were fairly well resolved. The total peak capacity of the comprehensive 2-D system could be as high as 2000. Yang *et al.* [65] developed a multi-dimensional counter-current chromatographic system and used it for the preparative separation of isorhamnetin, kaempferol, and quercetin from crude flavone aglycones of *Ginkgo biloba* L. and *Hippophae rhamnoides* L. Sheng *et al.* [66] developed a 2-D-LC/MS for analysis of the matrine alkaloids. Five alkaloids were detected and five compounds were detected from the gangliosides. Zou *et al.* [67 – 72] have performed much work on the separation of TCMs. They have constructed different effective comprehensive two-dimensional liquid chromatography modes and successfully applied them to the separation and identification of compounds in *Rhizoma chuanxiong* [67], *G. biloba* [68], honeysuckle [69], and *Psoralea corylifolia* [71]. Recently, 2-D LC, coupling a silica-bonded HSA column to a silica monolithic octadecylsilica column, was developed by Zou *et al.*, for analysis of the extract of *Rheum palmatum* L. [70]. A number of low-abundance components can be separated in a single peak from the HSA column after normalization of peak heights and six compounds were preliminarily identified according to their UV and MS spectra (Fig. 3). The performance of 2-D LC was further improved by using a silica monolithic column for the second-dimension separation [72]. The developed system was applied to anal-

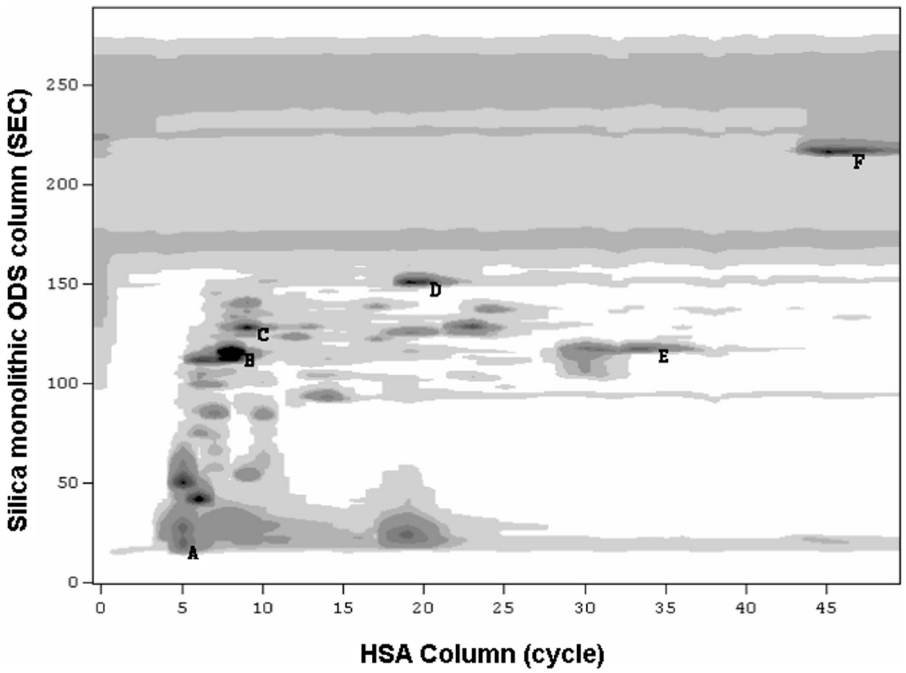

Figure 3. 2-D chromatogram of *R. palmatum* L. extract. Chromatographic conditions for the HSA column: isocratic elution with 15% ACN in 20 mM ammonium acetate buffer (pH 6.5); flow rate, 0.1 mL/min. Chromatographic conditions for the silica monolithic ODS column: linear gradient elution from 90% B (10% A) to 10% B (90% A) in 3.5 min, and then returning to the initial mobile phase and holding for 1.5 min for re-equilibration; flow rate, 3.0 mL/min; injection volume, 10 μL; detection wavelength, 250 nm. Cycle time for the second dimension is 5 min. Reprinted from [70] with permission.

ysis of the methanol extract of two umbelliferae herbs *Ligusticum chuanxiong* Hort. and *Angelica sinensis* (Oliv.) Diels. More than 117 components were detected by UV, APCI/MS, and MALDI-TOF/MS, and also 145 components could be detected by MALDI-TOF/MS alone.

4 Concluding remarks

A worldwide effort is underway to separate and characterize all proteins. To enhance the ability of separation and identification, especially toward the identification of protein modifications, MacCoss *et al.* [73] have combined proteolytic cleavage of different enzymatic activities for the generation of overlapping peptides. To obtain more molecular level information for the intact proteins, direct coupling of multidimensional protein separation technologies [74 – 76] with online trypsin reactor [77 – 79] for real-time and effective digestion of resolved proteins will enable integrated top-down and bottom-up proteomics in a single analysis. Separation, analysis, and screening of bioactive components in traditional Chinese medicines is also a very formidable task. However, it is our good fortune that the rapid development of modern analysis and separation methods offers a variety of means for elucidating the mysteries of proteomics and traditional Chinese medicines [80].

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