Journal of Chromatography A, 1218 (2011) 1749–1755

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A new method for chemical identification based on orthogonal parallel liquid chromatography separation and accurate molecular weight confirmation

Jing Zeng, Xiuli Zhang, Zhimou Guo, Jiatao Feng, Xingya Xue*, Xinmiao Liang*

Key Lab of Separation Science for Analytical Chemistry, Key Lab of Natural Medicine, Liaoning Province, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China

ARTICLE INFO

Article history:
Received 18 November 2010
Received in revised form 14 January 2011
Accepted 26 January 2011
Available online 1 February 2011

Keywords:
Orthogonal parallel separation
Retention time
Accurate molecular weight
Chemical identification
Flavonoids
Library searching

ABSTRACT

Recent advances in the theory and application of orthogonal LC separation have allowed for the establishment of a more effective method for the chemical identification of target compounds in complex samples, especially structurally similar compounds. In this study, a new chemical identification method based on orthogonal parallel separation and accurate molecular weight confirmation was developed. An orthogonal separation system consisting of an XTerra MS C_{18} column, a home-made Click OEG column, and a Click CD column was established for separation and identification. In addition, 82 flavonoids were selected as references, to be used for the construction of a library. Retention times of each reference flavonoid on each column and accurate molecular weights were recorded and imported into a searchable library as "tags" for the unknown screening. For the method validation, two complex mixtures, fractions of *Dalbergia odorifera* T. Chen and *Scutellaria baicalensis* Georgi, specifically, were separated and identified. In total, nine compounds were unequivocally identified by retention time and confirmation of accurate molecular weight, demonstrating that this method is suitable and efficient for the chemical identification of complex samples.

1. Introduction

Chemical identification, which identifies analytes as known chemical compounds based on matching properties, greatly impacts international trade, economy, R&D, justice, public safety, social and personal life, and sport. Analytical methods and strategies have been thoroughly reviewed by Milman [1] and Valcarcel et al. [2]. Infrared spectra (IR) and ultraviolet spectra (UV) distinguish structurally similar compounds with questionable specificity, and have gradually been replaced by methods of GC or LC tandem mass spectrometry, which demonstrate superior separation and detection ability. GC hyphenated with electron impact ionization mass spectrometry (GC–EI-MS) has been a powerful method for chemical identification, due to its stable product ions fragmentation, searchable MS/MS library, and retention index confirmation. However, GC–MS shows poor detection for polar, thermo-labile and high-mass molecules [3,4]. In contrast, liquid chromatography hyphenated with mass spectrometry (LC–MS) seems to be suitable for the universal analysis of target compounds in complex samples, without additional sample derivatization. Moreover, the LC–MS method allows for the selection of various separation modes (RPLC, NPLC, IEC, etc.) [5] and the acquisition of structural information.

Chemical identification of target compounds in complex samples with LC–MS is still a great challenge for chemists, however, even though this method has previously been used for rapid chemical identification in the field of natural products [6], drugs [7,8], forensic and clinical toxicology [9–11] and environment [12]. Due to the limited peak capability of unidimensional liquid chromatography, many compounds may elute simultaneously during LC separation, preventing their differentiation according to retention time. In addition, structural isomers, especially conformational isomers from complex samples eluted simultaneously during LC, may fragment highly similar mass spectra [13]. Finally, a universal MS/MS library that can be searched to rapidly identify analytes from complex samples is still lacking. Therefore, retention times and mass spectral matching provided by unidimensional LC–MS is still insufficient for the unambiguous identification of complex samples.

In recent decades, there has been increasing interest in orthogonal methods of separation, which could potentially separate overlapping peaks. Both parallel unidimensional LC (1D-LC) and two-dimensional LC (2D-LC) can be used for separation, either by using two or more significantly dissimilar columns or by changing chromatographic conditions [14]. Methods involving 1D-LC have allowed for the rapid screening of compounds, and have

* Corresponding authors. Tel.: +86 411 84379519; fax: +86 411 84379539.
E-mail addresses: xuexy@dicp.ac.cn (X. Xue), liangxm@dicp.ac.cn (X. Liang).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.01.079

Table 1
Names of the 82 references used in this study.

No.	Name	No.	Name
1	Isocarthamidin	42	Genistein
2	Scutellarin	43	Glucoliquiritin apioside[*3]
3	Luteolin	44	Glucoliquiritin apioside[*3]
4	Apigenin	45	Vicenin-2
5	Acacetin-7-rutinoside	46	Liquiritin
6	Apigenin-5-O-β-glucoside	47	Glycyroside
7	Apigenin-7-O-glucuronide	48	Licuraside
8	4′-Hydroxy-wogonin-7-O-β-D-glucuronide[*1]	49	Ononin
9	4′-Hydroxy-wogonin-7-O-β-D-glucuronide[*1]	50	Isoliquiritin
10	Sinensetin	51	Violanthin
11	Nobiletin	52	Isoviolanthin
12	3,3′,4′,5,6,7,8-Heptamethoxyflavone	53	Hydroxysafflor yellow A
13	4′,5,6,7-Tetramethoxyflavone	54	Rutin
14	5-demethyl-nbiletin	55	Quercetin
15	Chyrsin-6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranoside	56	Kaempferol
16	Chyrsin-8-C-α-L-arabinopyranosyl-6-C-β-D-glucopyranoside	57	Kaempferol-3-O-[2-O-(6-O-E-ferloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside
17	Baicalin	58	Quercetin-3-O-[2-O-(6-O-E-ferloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside
18	Wogonoside	59	4′-Hydroxywogonin
19	Wogonin	60	Mangiferin
20	Hesperitin	61	2′,4′-Dimethoxy-3,7- dihydroxyisoflavanone[*4]
21	Hesperidin	62	2′,4′-Dimethoxy-3,7- dihydroxyisoflavanone[*4]
22	Naringenin	63	Alpinetin[*5]
23	Naringin[*2]	64	Alpinetin[*5]
24	Naringin[*2]	65	3ĭ-O-Methylviolanone
25	5-Hydroxypuerarin	66	Sativanone
26	Puerarin	67	4′-Methoxy-2′,3,7-trihydroxyisoflavanone[*6]
27	3′-Methoxypuerarin	68	4′-Methoxy-2′,3,7-trihydroxyisoflavanone[*6]
28	4′-Methoxydadzin	69	Liquoritigenin
29	Daidzin	70	Violanone[*7]
30	Daidzein	71	Violanone[*7]
31	Puerarin-6″-apioside	72	Vestitone
32	Genistin	73	Formononetin
33	Formononetin-8-C-apiosyl (1 → 6)-glucoside	74	3′,4′,7-Trihydroxyflavanone[*8]
34	Formononetin 8-C-xylosyl (1 → 6)-glucoside	75	3′,4′,7-Trihydroxyflavanone[*8]
35	Bellidifolin-8-O-glucoside	76	Pinocembrin[*9]
36	1,7,8-Trihydroxy-3-methoxy-xanthonoid-7-O-xylosyl (1 → 2) xyloside	77	Pinocembrin[*9]
37	1,7,8-Trihydroxy-3-methoxy-xanthonoid-7-O-rhamnosyl (1 → 2) xyloside	78	Isoliquiritigenin
38	1-Hydroxy-3,7,8-trimethoxy-xanthonoid-1-O-pimeoeroside	79	Butein
39	1-Hydroxy-2,3,4,5-tetramethoxyxanthonoid -1-O-pimeoeroside	80	Isoquercitrin
40	1,5,8-Trihydroxy-3-methoxyxanthonoid	81	Genkwanin
41	1-Hydroxy-3,7,8-trimethoxyxanthonoid	82	Neohesperidin

[*n] ($n = 1 \sim 9$): it means nine pairs of stereoisomers (8-9, 23-24, 43-44, 61-62, 63-64, 67-68, 70-71, 74-75, 76-77).

been widely used for impurity evaluation in the production of active pharmaceutical ingredients [14–17]. Recently, Dumarey et al. reported that a parallel 1D-LC system, composed of a HC-SO_3 column coupled to a BetabasicPhenyl column, a HC-OH column and a StableBond C_{18} column, respectively, showed excellent orthogonal separation for 25 drugs [18]. Furthermore, 2D-LC can be used for the separation and identification of extremely complex samples, which is difficult to accomplish with 1D-LC due to limited peak capacity. Many 2D-LC systems were established by changing separation mode and column type [19–21], or mobile phase pH [22,23]. Additionally, a few 2D-LC systems were developed composed of a single column operated under different modes [24–26]. Because such systems are highly orthogonal, unresolved compounds in one chromatogram would likely be separated in the second chromatogram [18]. Retention times on the two chromatograms could be used as "tags" for the differentiation of unresolved compounds, since retention time under a given condition is a specific property of a compound. The "tags" can be imported into a library, and the retention times of unknowns and references could be automatically matched, which would increase identification efficiency.

In this study, a commercial C_{18} column and two novel homemade columns (Click OEG column [27] and Click CD column [28]) differing significantly in chromatographic selectivity, were employed as an orthogonal parallel separation system for the acquisition of reference retention times and the identification of unknown analytes. Simultaneously, a searchable library was developed to automatically match retention times between references and unknowns. In addition, accurate molecular weight confirmation was used to increase the rate of positive identification. Compared with the unidimensional LC–MS method, the method established in this study is more effective for the chemical identification of target compounds in complex samples.

2. Experimental

2.1. Materials and reagents

The three columns used in this study are listed as follows: XTerra 5 μm MS C_{18} (150 mm × 2.1 mm, waters), which will subsequently be referred to as C_{18}, homemade 5 μm Click OEG (150 mm × 4.6 mm) [27], and homemade 5 μm Click CD (150 mm × 4.6 mm) [28], which will subsequently be referred to as the OEG column and CD column, respectively. All references were

Table 2
Separation conditions for the three columns in this study.

Columns	Mobile phases	Gradient	Inj. vol. (μL)	Flow rate (ml/min)
C_{18}	A: 0.2% formic acid–H_2O; B: 0.2% formic acid–ACN	(95:5) → (30:70) (A:B) (15 min)	1	0.3
OEG	A: 0.2% formic acid–H_2O; B: 0.2% formic acid–ACN	(95:5) → (40:60) (A:B) (30 min)	10	1
CD	A: H_2O; B: ACN; C: 100 mM ammonium formate	(65:5:30) → (30:40:30) (A:B:C) (30 min)	10	1

purified in our laboratory and identified with NMR and MS. Eighteen groups of isomers (69–76–77–78, 6–25–32, 4–42, 63–64, 8–9, 51–52, 22–74–75–79, 19–81, 2–3, 15–16–31, 20–41–67–68, 33–34, 23–24, 61–62–70–71, 46–50, 26–29, 21–82, 43–44) were included in the reference list (Table 1).

HPLC grade acetonitrile was purchased from TEDIA (Fairfield, USA), formic acid and ammonium formate were purchased from Acros (Cambridge, USA), and water was prepared with a Milli-Q system (MA, USA).

2.2. LC–MS conditions

Determination of retention times of references and unknowns was carried out on a Waters Alliance HPLC system, consisting of a 2695 HPLC pump and a 2996 photodiode array detection system. Data acquisition and processing were conducted using Waters Empower software. Column temperature was maintained at 30 °C and equilibrium time was 15 min for the three columns. Other chromatographic conditions are detailed in Table 2.

MS experiments were performed on a Waters Micromass Q-TOF system (Milford, MA, USA) operated in positive and negative ion mode. The operation parameters for positive and negative ion mode were the same and are listed as follows: capillary: 2 kV; cone voltages: 20 V; desolvation temperature: 300 °C; source temperature: 100 °C; cone gas: 50 L/h; desolvation gas: 1000 L/h. Leucine-enkephalin was used as lock mass through the Lockspray interface. The mass scan range was set to 50–850 m/z with a scan time of 0.3 s and an interscan delay of 0.1 s. Masslynx was employed as the chemstation for data acquisition.

2.3. Sample preparation

Two samples, ethyl acetate fractions of *Scutellaria baicalensis* Georgi and *Dalbergia odorifera* T. Chen, were prepared in the same way for use in method validation. Preparation procedures are listed as follows: 100 g of *S. baicalensis* Georgi (*D. odorifera* T. Chen) was extracted in water twice with reflux times of 2 h and 1.5 h, respectively. The decoctions were combined and then concentrated with a spray dryer. The resulting 1.5 g of fine powder was dissolved in 4.5 ml of water, and then 10 ml of 95% ethanol was added and deposited for 24 h. The supernatant was concentrated to 1.5 ml, and then the solution was extracted in 5 ml of ethyl acetate and 2.5 ml of ethyl acetate continuously. The ethyl acetate fraction was collected and dried with a spray dryer. For analysis, the two ethyl acetate fractions were each dissolved in methanol to approximately 1 mg ml^{-1}. The injection volume of both fractions was 5 μL for the OEG column and CD column, and 1 μL for the C_{18} column.

2.4. Software and interval settings

The software used for screening was developed in-house with Microsoft Visual Basic 6.0, and was run in Windows XP Professional on a personal computer. The data for references and unknowns was imported into this software to create a searchable library. Several parameters were also set, including retention time thresholds for the C_{18} column, OEG column and CD column, as well as a threshold for accurate molecular weight. In this study, retention time thresholds for the C_{18} column, OEG column and CD column were set at 0.10 min, 0.3 min and 0.10 min, respectively, based on results from the reproducibility test (see Section 3.4). The accurate molecular weight interval was set at 20 ppm. The matching results were listed once the process was finished.

3. Results and discussion

3.1. Reference selection for library construction

Considering growing interest in natural products, we aimed to develop a rapid and universal method for the chemical identification of natural products. Flavonoids are widely distributed in the plant kingdom and show a wide range of bioactivity. In this study, 82 flavonoids, including flavones, flavonol, flavonones, flavanonol and chalcone, were selected as references for the construction of the library. These references, including eighteen groups of isomers, were all purified in our laboratory and identified by NMR and MS spectroscopy. The structurally similar references can be used to evaluate the orthogonal separation system. Additionally, some of these reference compounds are commonly occurred in many plants, including hesperdin and naringin [29], which bodes well for practical application. Therefore, these references were used to create a representative and applicable library for the identification of flavonoids.

3.2. Selection of columns

In this section, we aimed to establish a highly orthogonal separation system for the differentiation of unresolved compounds or structurally similar analytes by using several different columns. It was necessary for the columns to afford different selectivities for separation, so that fairly similar analytes could be distinguished from one another. Different stationary phases with different bonded functional groups provide different selectivities [5,30]. Thus, co-eluted compounds on one column could likely be separated on other columns. Overall, retention times on different columns can be recorded and used as tags for the chemical identification of analytes, since retention times reflect the structural characteristics of analytes [2].

C_{18} is one of the most common stationary phases for the separation of compounds of medium to weak polarity due to its high efficiency, stability and reproducibility. A commercial C_{18} column (XTerra MS C_{18} column) was utilized for the separation system. Recently in our laboratory, several newly developed stationary phases have been synthesized and evaluated for specific selectivity [27,28]. OEG was shown to be a novel stationary phase with good performance for the separation of phenyl mixtures and *Lamiophlomis rotate* [27]. Additionally, a two-dimensional reversed-phase/reversed-phase liquid chromatography system (2D-RP/RP-LC), coupled with OEG and C_{18}, was set up and demonstrated higher orthogonality than C_{18}/CN and C_{18}/C_{18} [31]. CD is also a type of home-made stationary phase, which has demonstrated HILIC/RPLC mixed-mode retention behavior [28] and different selectivity for the separation of *herba hedyotis diffusae* [24]. Thus, a highly orthogonal separation system, consisting of C_{18} and CD, and operated under RP and HILIC mode, respectively, was successfully established for the separation of *Carthamus tinstorius Linn*. [32]. The parallel orthogonal separation

Table 3
Retention time differences of 38 pairs of references on C_{18}, OEG and CD column.

Pairs of references	$\Delta t_{R(C18)}$[a]	$\Delta t_{R(OEG)}$[b]	$\Delta t_{R(CD)}$[c]	Pairs of references	$\Delta t_{R(OEG)}$	$\Delta t_{R(C18)}$	$\Delta Dt_{R(CD)}$	Pairs of references	$\Delta t_{R(CD)}$	$\Delta t_{R(C18)}$	$\Delta t_{R(OEG)}$
75–79	0	2.57	2.31	10–20	0	1.01	0.60	66–72	0.01	1.86	2.41
17–28	0	3.32	8.39	38–82	0.01	0.50	0.43	2–71	0.01	1.52	0.41
20–63	0	0.93	1.31	28–57	0.01	0.94	5.78	42–73	0.01	1.20	0.65
38–39	0.01	1.05	2.89	2–36	0.01	1.42	2.48	28–32	0.01	1.39	1.42
46–80	0.01	0.45	2.70	41–81	0.03	1.87	1.02	63–74	0.01	2.59	3.48
26–60	0.01	0.69	2.49	6–80	0.03	0.09	2.16	1–59	0.01	2.05	3.97
2–49	0.02	1.01	0.49	–	–	–	–	10–49	0.02	3.26	3.76
42–72	0.02	0.81	0.7	–	–	–	–	9–14	0.02	6.46	7.06
58–32	0.03	0.40	6.93	–	–	–	–	22–69	0.02	1.22	2.30
12–77	0.02	2.04	1.68	–	–	–	–	61–75	0.02	2.03	1.51
4–59	0.03	0.63	3.28	–	–	–	–	53–62	0.03	4.92	2.68
22–42	0.02	0.11	3.05	–	–	–	–	26–54	0.03	1.17	3.62
51–52	0.02	0.26	0.21	–	–	–	–	16–37	0.03	3.22	6.23
26–31	0.03	0.26	1.22	–	–	–	–	8–68	0.03	0.68	0.83
–	–	–	–	–	–	–	–	78–81	0.03	1.78	1.34
–	–	–	–	–	–	–	–	25–44	0.03	0.37	1.04
–	–	–	–	–	–	–	–	47–75	0.02	0.03	0.05
–	–	–	–	–	–	–	–	11–19	0.01	0.13	1.60

[a] $\Delta t_{R(C18)}$ means absolute difference of retention times between two references on C_{18} column (min).
[b] $\Delta t_{R(OEG)}$ means absolute difference of retention times between two references on OEG column (min).
[c] $\Delta t_{R(CD)}$ means absolute difference of retention times between two references on CD column (min).

system, consisting of C_{18}, OEG and CD allowed for large discrepancies in the retention times of the 82 reference flavonoids. Thus, unresolved compounds or structurally similar compounds could be distinguished according to the retention times on the different columns of this system.

3.3. Orthogonal separation

In order to create the library, the retention times of each reference on the C_{18}, OEG and CD columns were determined (retention times of all references are detailed in supporting information). All references were analyzed on these three columns under three different chromatographic conditions, which simplified the optimization process of unknowns. Some references showed the same retention time on one column, but could be differentiated on other column(s). As shown in Table 3, eleven pairs of references (75–79, 17–28, 20–63, 38–39, 46–80, 26–60, 2–49, 42–72, 58–32, 12–77, 4–59) eluted simultaneously on the C_{18} column ($\Delta t_{R(C18)} \leq 0.03$ min), but were well separated on the OEG column ($\Delta t_{R(OEG)} \geq 0.40$ min) and CD column ($\Delta t_{R(CD)} \geq 0.49$ min). Five pairs of references (10–20, 38–82, 28–57, 2–36, 41–81) eluted simultaneously on the OEG column ($\Delta t_{R(OEG)} \leq 0.03$ min), but were well separated on the C_{18} column ($\Delta t_{R(C18)} \geq 0.50$ min) and the CD column ($\Delta t_{R(CD)} \geq 0.43$ min). Similarly, references unresolved on the CD column could be separated on the C_{18} column and the OEG column. Moreover, the CD column was shown to be capable of chiral separation (see supporting information), which is in accordance with our previous study [28]. It is noteworthy that four pairs of references (22–42, 51–52, 26–31 and 6–80), including a pair of isomers (51–52), showed similar retention behavior on the C_{18} column ($\Delta t_{R(C18)} \leq 0.03$ min), but were well resolved on the CD column ($\Delta t_{R(CD)} \geq 0.21$ min). As discussed above, these stationary phases with different chemistry afford different selectivity, allowing analytes with minor differences to be distinguished. Theoretically, a greater number of columns with different selectivities could be used to provide even more accurate identification results, since retention times reflect specific characteristics of analytes.

In order to visually evaluate the orthogonality of this separation system, correlation coefficients (r^2) between retention factors (k) for the 82 references on the C_{18}, OEG, and CD columns were calculated. Lower correlation coefficients correspond to higher orthogonality. As shown in Fig. 1, the correlation coefficient between C_{18} and CD columns ($r^2 = 0.495$) is lower than the correlation coefficients between the C_{18} and OEG columns ($r^2 = 0.887$) or between the OEG and CD columns ($r^2 = 0.688$). The orthogonality primarily contributed to the change of stationary phase, orthogonality of the developed system can afford enough identification power for the fairly similar compounds based on the discussion above.

3.4. Reproducibility of retention times

Reproducibility of retention times is critical for effective library searching and is controllable under strictly defined operation conditions, such as column temperature, contents of mobile phase additives, pH, flow rate and equilibrium time [33]. In order to evaluate reproducibility, ten references, divided into three subgroups for the three aforementioned columns, were used as test probes over a period of ten days. As shown in Table 4, probes (4, 39, 41, 54, 65 and 76) were used to evaluate the reproducibility of the C_{18} column, and the maximum deviation of retention time ($\Delta t_{R(max)}$) was 0.08 min. Based on this result, the threshold for this column was set at 0.10 min for the library searching process. Probes (12, 41, 46 and 54) were also used to evaluate the reproducibility of the CD column. The $\Delta t_{R(max)}$ was 0.09 min, so the threshold for the CD column was also set at 0.10 min. Finally, probes (4, 41, 43, 53 and 54) were used to evaluate the reproducibility of the OEG column, and the $\Delta t_{R(max)}$ was 0.27 min. So, the threshold for the OEG column was set at 0.30 min. Although the deviation on OEG column is larger than that on C_{18} and CD column, it is still tolerable, since thresholds as large as 1 min have been reported in previous studies [11].

3.5. Construction of the multiple retention LC–MS library

As discussed above, the three retention times obtained in this study were used as "tags" for the chemical identification of analytes. MS/MS matching is a traditional and powerful method for qualitative analysis, although the pathways and intensities of fragments varied with the use of different types of instruments and operator parameters [7,9,34–36]. In order to increase the success rate of positive identification, accurate molecular weight, which is stable and reproducible when "soft" ionization technologies and high-resolution analyzers are employed, was only used for confirmation in this work. Take references 47–75 as an example (see Table 3): these references were almost overlapping on the C_{18}

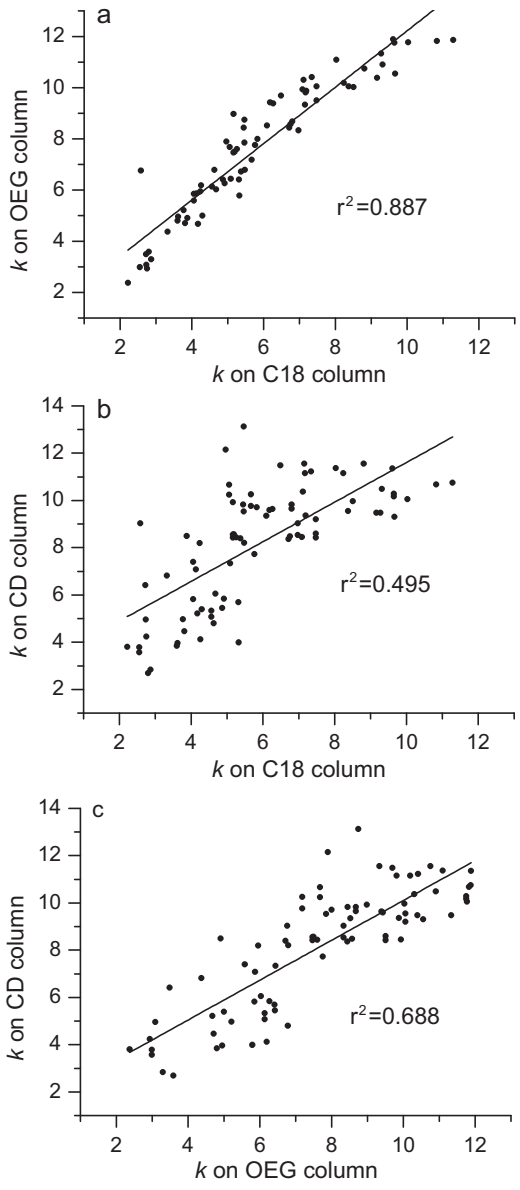

Fig. 1. Correlation coefficients between retention factors for the 82 reference on the C_{18}/OEG column (a), C_{18}/CD column (b) and OEG/CD column (c). Chromatographic conditions are detailed in Section 2.2.

column ($\Delta t_{R(C18)}$ = 0.03 min), the OEG column ($\Delta t_{R(OEG)}$ = 0.05 min) and the CD column ($\Delta t_{R(CD)}$ = 0.02 min), even though a highly orthogonal separation system was employed. However, they were clearly differentiated through accurate molecular weight confirmation (MW_{47} = 561.1608, MW_{75} = 272.0685).

Additionally, using these three retention times with accurate molecular weight confirmation demonstrates more accurate identification than unidimensional retention time combined with accurate molecular weight confirmation. For example, two pairs of isomers (4–42 and 15–16) cannot be differentiated ($\Delta t_{R(C18)} \leq 0.07$ min, deviation = 0 ppm) through the use of a unidimensional C_{18} column coupled with accurate molecular weight confirmation. However, these isomers can be resolved on the OEG column ($\Delta t_{R(OEG)} \geq 0.33$ min) and CD column ($\Delta t_{R(CD)} \geq 1.32$ min). So, using retention times on multiple columns combined with accurate molecular weight confirmation is a reliable tool for unequivocal identification.

For library construction, the retention times of references were all measured triplicate, and average retention times were used as "tags" for chemical identification, which reduced systematic deviation. Three retention times on three columns and accurate molecular weight of references were imported and stored in the library as reference data. Then, the retention times and accurate molecular weights of unknowns, which were measured under the same operation conditions as that for references' data acquisition, were imported for the matching process. The searchable library can be used to identify compounds directly, which avoids re-analysis of target references and thus increases identification efficiency.

3.6. Application

In this work, parallel LC was employed to test and validate our method. Two complex mixtures, fractions of *D. odorifera* T. Chen (fraction A) and *S. baicalensis* Georgi (fraction B) were chosen as unknowns, and were identified with the use of two columns (C_{18} and OEG) and three columns (C_{18}, OEG and CD), respectively. As shown in Fig. 2, fraction A was separated on the C_{18} and OEG columns. Different elution order on the two columns was observed. Retention times of all peaks (signal to noise ≥ 3) from the two columns were imported into the library. Automatic matching with references was performed, yielding eight hits as a search result, as shown in Table 5. Similarly, fraction B was successfully separated and identified on the C_{18}, OEG and CD columns, which was shown in Fig. 3. After automatic searching, one compound was listed as a result. Retention times of the identified compound were 11.53 min on the C_{18} column, 21.09 min on the OEG column and 17.89 min on the CD column. These retention times matched reference 19 well ($\Delta t_{R(C18)}$ = 0.02 min, $\Delta t_{R(OEG)}$ = 0.13 min, $\Delta t_{R(CD)}$ = 0.07 min). Furthermore, this compound possessed the same molecular weight as reference 19 (deviation = 2.1 ppm). This reference matching identi-

Table 4
Relative standard deviation (RSD%) and maximum retention time deviation ($\Delta t_{R(max)}$) of 10 probes on C_{18}, OEG and CD column, respectively.

References	C_{18} (n = 22)		OEG (n = 32)		CD (n = 20)	
	RSD%	$\Delta t_{R(max)}$[a]	RSD%	$\Delta t_{R(max)}$	RSD%	$\Delta t_{R(max)}$
4	0.12	0.04	0.4	0.26	–	–
12	–	–	–	–	0.11	0.07
39	0.15	0.05	–	–	–	–
41	0.13	0.08	0.36	0.27	0.08	0.06
43	–	–	0.64	0.16	–	–
46	–	–	–	–	0.13	0.06
53	–	–	0.45	0.22	–	–
54	0.18	0.05	0.60	0.23	0.27	0.09
65	0.12	0.06	–	–	–	–
76	0.13	0.07	–	–	–	–

[a] $\Delta t_{R(max)}$ maximum retention time deviation ($\Delta t_{R(max)} = t_{R(max)} - t_{R(min)}$) (min).

Table 5
Identification results of the ethyl acetate extraction of *Dalbergia odorifera* T. Chen.

Unknowns	$t_{R(C18)}$[a]	$t_{R(OEG)}$[b]	AMW[c]	$\Delta t_{R(C18)}$[d]	$\Delta t_{R(OEG)}$[e]	Deviation[f]	Identification
P1	6.91	14.37	272.0640	0.01	0.02	16.5	3′,4′,7-Trihydroxyflavanone
P2	7.47	13.87	302.0768	0	0.05	7.3	4′-Methoxy-2′,3,7-trihydroxy-isoflavanone
P3	8.74	16.43	316.0911	0	0.03	11.4	Violanone
P4	9.19	17.56	268.0824	0.06	0.01	6.3	Vestitone
P5	10.13	20.55	256.0719	0.02	0.02	6.6	Isoliquiritigenin
P6	10.65	18.74	330.1079	0	0.01	7.3	3′-O-methylviolanone
P7	10.99	19.98	300.0967	0	0	10.3	Sativanone
P8	11.93	21.67	256.0698	0	0.01	14.8	Pinocembrin

[a] $t_{R(C18)}$ means measured retention times on C_{18} column (min).
[b] $t_{R(OEG)}$ means measured retention times on OEG column (min).
[c] AMW means measured accurate molecular weight (amu).
[d] $\Delta t_{R(C18)}$ means absolute difference of retention times between unknowns and references on C_{18} column (min).
[e] $\Delta t_{R(OEG)}$ means absolute difference of retention times between unknowns and references on OEG column (min).
[f] Deviation means accurate mass weight deviation between theoretical mass weight and measured mass weight (ppm).

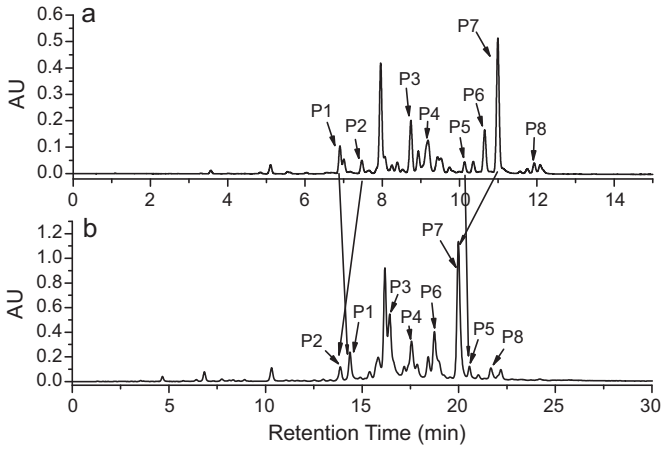

Fig. 2. Separation of the ethyl acetate extraction of *Dalbergia odorifera* T. Chen on the C_{18} column (a) and OEG column (b). Chromatographic conditions are detailed in Section 2.2.

fied the peak eluted at 11.53 min on the C_{18} column as wogonin. As mentioned, the nine identified compounds were previously purified from *D. odorifera* T. Chen and *S. baicalensis* Georgi, for use as library references. Results were reasonable and provided evidence that the developed method can provide accurate chemical identification.

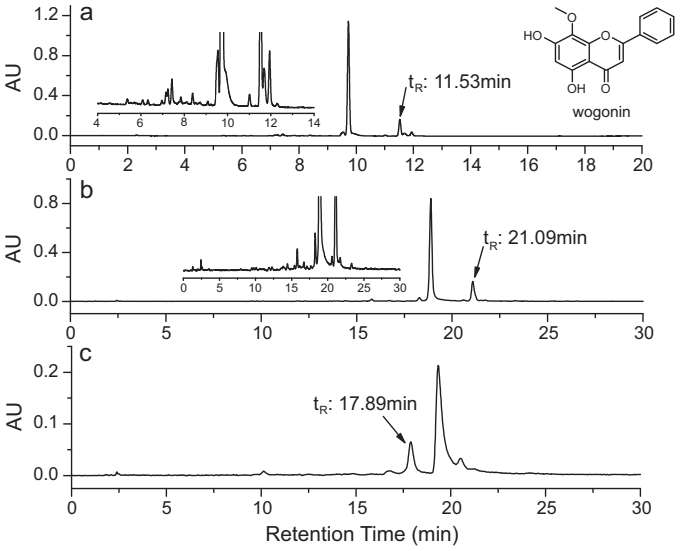

Fig. 3. Separation of the ethyl acetate extraction of *Scutellaria baicalensis* Georgi on the C_{18} column (a), OEG column (b), and CD column (c). Chromatographic conditions are detailed in Section 2.2.

4. Conclusion

A new method for chemical identification of target compounds in complex samples was developed based on parallel orthogonal separation and accurate molecular weight confirmation. Unresolved compounds or structurally similar compounds with similar product ion fragments can be unequivocally identified with this LC-based method. A searchable library was simultaneously created by compiling the retention times and accurate molecular weights of 82 flavonoids. Using this library, eight compounds in the *D. odorifera* T. Chen extraction and one compound in the *S. baicalensis* Georgi extraction were successfully identified. This method was shown to be effective for the identification of complex samples. Moreover, the screening results obtained through this library can be validated with MS/MS fragments if necessary. Although the feasibility of the identification method established in this study was validated, throughput still must be improved for practical purposes. Ultra-performance LC and advanced LC equipment with automatic column switching functions could be good choices for improvement of throughput.

As discussed above, a universal library could likely be established if enough references were obtained and standard chromatographic conditions were adopted. Additionally, this method of chemical identification could potentially be used to screen veterinary drug residues, which are currently under investigation in our laboratory.

Acknowledgements

This work was supported by National Key Technology R&D Program in the 11th 5-year Plan of China (No. 2009BADB9B02), Major National Sci-Tech Projects (2009ZX09301-012 and 2009ZX09313-003) and Project of National Science Foundation of China (30801513). Thanks to Lillian Nordahl for the manuscript revision.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.01.079.

References

[1] B.L. Milman, Trac-Trends Anal. Chem. 24 (2005) 493.
[2] M. Valcarcel, S. Cardenas, B.M. Simonet, C. Carrillo-Carrion, J. Chromatogr. A 1158 (2007) 234.
[3] P. Marquet, Ther. Drug Monit. 24 (2002) 125.
[4] L. Rivier, Anal. Chim. Acta 492 (2003) 69.

[5] P. Jandera, J. Sep. Sci. 29 (2006) 1763.
[6] J.S. Lee, D.H. Kim, K.H. Liu, T.K. Oh, C.H. Lee, Rapid Commun. Mass Spectrom. 19 (2005) 3539.
[7] M. Pavlic, K. Libiseller, H. Oberacher, Anal. Bioanal. Chem. 386 (2006) 69.
[8] X.M. Chong, C.Q. Hu, Chromatographia 68 (2008) 759.
[9] S. Dresen, M. Gergov, L. Politi, C. Halter, W. Weinmann, Anal. Bioanal. Chem. 395 (2009) 2521.
[10] K. Pihlainen, E. Sippola, R. Kostiainen, J. Chromatogr. A 994 (2003) 93.
[11] H.C. Liu, R.H. Liu, D.L. Lin, H.O. Ho, Rapid Commun. Mass Spectrom. 24 (2010) 75.
[12] M. Ibanez, J.V. Sancho, O.J. Pozo, W. Niessen, F. Hernandez, Rapid Commun. Mass Spectrom. 19 (2005) 169.
[13] C. Wagner, M. Sefkow, J. Kopka, Phytochemistry 62 (2003) 887.
[14] J. Pellett, P. Lukulay, Y. Mao, W. Bowen, R. Reed, M. Ma, R.C. Munger, J.W. Dolan, L. Wrisley, K. Medwid, N.P. Toltl, C.C. Chan, M. Skibic, K. Biswas, K.A. Wells, L.R. Snyder, J. Chromatogr. A 1101 (2006) 122.
[15] E. Van Gyseghem, M. Jimidar, R. Sneyers, D. Redlich, E. Verhoeven, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 1074 (2005) 117.
[16] G. Xue, A.D. Bendick, R. Chen, S.S. Sekulic, J. Chromatogr. A 1050 (2004) 159.
[17] E. Van Gyseghem, S. Van Hemelryck, M. Daszykowski, F. Questier, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 988 (2003) 77.
[18] M. Dumarey, Y.V. Heyden, S.C. Rutan, Anal. Chem. 82 (2010) 6056.
[19] Y.L. Wei, T. Lan, T. Tang, L.Y. Zhang, F.Y. Wang, T. Li, Y.P. Du, W.B. Zhang, J. Chromatogr. A 1216 (2009) 7466.
[20] H.Z. Tian, J. Xu, Y.F. Guan, J. Sep. Sci. 31 (2008) 1677.
[21] S. Ma, L.X. Chen, G.A. Luo, K.N. Ren, J.F. Wu, Y.M. Wang, J. Chromatogr. A 1127 (2006) 207.
[22] C.X. Song, M.L. Ye, G.H. Han, X.N. Jiang, F.J. Wang, Z.Y. Yu, R. Chen, H.F. Zou, Anal. Chem. 82 (2010) 53.
[23] J. Zhang, Y. Jin, Y.F. Liu, Y.S. Xiao, J.T. Feng, X.Y. Xue, X.L. Zhang, X.M. Liang, J. Sep. Sci. 32 (2009) 2084.
[24] J.T. Feng, Z.M. Guo, H. Shi, J.P. Gu, Y. Jin, X.M. Liang, Talanta 81 (2010) 1870.
[25] X.D. Geng, C.Y. Ke, G. Chen, P. Liu, F. Wang, H.Q. Zhang, X. Sun, J. Chromatogr. A 1216 (2009) 3553.
[26] J. Urban, V. Skerikova, P. Jandera, R. Kubickova, M. Pospisilova, J. Sep. Sci. 32 (2009) 2530.
[27] Z.M. Guo, Y.F. Liu, J.Y. Xu, Q. Xu, X.Y. Xue, F.F. Zhang, Y.X. Ke, X.M. Liang, A.W. Lei, J. Chromatogr. A 1191 (2008) 78.
[28] Z.M. Guo, Y. Jin, T. Liang, Y.F. Liu, Q. Xu, X.M. Liang, A.W. Lei, J. Chromatogr. A 1216 (2009) 257.
[29] J.T. Dwyer, J.J. Peterson, Public Health Nutr. 5 (2002) 925.
[30] P. Jandera, M. Halama, L. Kolarova, J. Fischer, K. Novotna, J. Chromatogr. A 1087 (2005) 112.
[31] Y.M. Liu, Z.M. Guo, Y. Jin, X.Y. Xue, Q. Xu, F.F. Zhang, X.M. Liang, J. Chromatogr. A 1206 (2008) 153.
[32] Y.M. Liu, X.Y. Xue, Z.M. Guo, Q. Xu, F.F. Zhang, X.M. Liang, J. Chromatogr. A 1208 (2008) 133.
[33] F. Pragst, M. Herzler, B.T. Erxleben, Clin. Chem. Lab. Med. 42 (2004) 1325.
[34] H. Oberacher, M. Pavlic, K. Libiseller, B. Schubert, M. Sulyok, R. Schuhmacher, E. Csaszar, H.C. Kofeler, J. Mass Spectrom. 44 (2009) 485.
[35] H. Oberacher, M. Pavlic, K. Libiseller, B. Schubert, M. Sulyok, R. Schuhmacher, E. Csaszar, H.C. Kofeler, J. Mass Spectrom. 44 (2009) 494.
[36] C. Hopley, T. Bristow, A. Lubben, A. Simpson, E. Bul, K. Klagkou, J. Herniman, J. Langley, Rapid Commun. Mass Spectrom. 22 (2008) 1779.