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Technical Report

Characterization of *Glycyrrhiza Glabra* Extract by Comprehensive Two-dimensional Liquid Chromatography-mass Spectrometry Using Multi-segmented Shift Gradients in the Second Dimension

LC×LC analysis of the *Glycyrrhiza glabra* extract

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Abstract:

Metabolic profiling of *Glycyrrhiza glabra* using comprehensive two-dimensional liquid chromatography (LC×LC) coupled with photodiode array (PDA) and mass spectrometry (MS) detection is described. The separation was conducted under reversed-phase conditions, using a combination of first dimension (^{1}D) 150 mm microbore cyano column utilising 2.7 µm diameter (dp) particles, and second dimension (^{2}D) 50 mm superficially porous octadecylsilica column with 2.7 µm dp particles. A multi-segmented shift gradient (MSG) for the 2D separation was developed, and the orthogonality achieved was compared with other modes of gradients, such as full in-fraction, and shift gradient systems. Results demonstrated a significant expansion of metabolic coverage using MSG in 2D, providing the highest measure of orthogonality compared to other gradient modes., leading to the identification of ca. 120 compounds.

Keywords: Comprehensive 2D liquid chromatography, *Glycyrrhiza glabra*, Food analysis

1. Introduction

The genus Glycyrrhiza, comprising of more than 28 species belonging to the Fabaceae family, is one of the oldest and widely used herbal medicines in the world, and is documented in various Asian and European pharmacopoeias. Clinically, licorice has been reported to exhibit a variety of pharmacological activities, including anti-cancer, anti-viral, anti-oxidative, anti-diabetic, anti-microbial, anti-inflammatory and other forms of biological activities. Licorice consists of significant quantities of secondary compounds, particularly triterpene saponins, and phenolic compounds such as flavones, isoflavones, flavanones, and chalcones, which are considered to be responsible for the bioactivities of licorice.

The metabolic profiling of *G. glabra* is normally carried out either using high performance liquid chromatography (HPLC) or ultra-high performance LC (UHPLC) with photodiode array (PDA) and/or MS detection. However, the physiochemical diversity of secondary metabolites (e.g., polyphenols and saponins) having similar retention behaviour tend to exceed the peak capacity of a single separation system. The heterogeneity of isobaric molecules also limits the identification based solely on MS data. The coupling of different separation mechanisms (e.g., hydrophobicity, polarity, size and charge) to achieve a potentially "orthogonal" separation in comprehensive two-dimensional liquid chromatography (LC×LC) can be of great aid.

In this contribution a systematic evaluation of RP-LC×RP-LC coupled with PDA and MS detection for the untargeted metabolic profiling of *G. glabra* extract is reported. The separation was conducted using a combination of first dimension (^{1}D) microbore cyano column, and second dimension (^{2}D) superficially porous C18 column. Different 2D gradient modes were investigated to understand separation behaviour of different compound classes in RP-LC×RP-LC analysis. A new ^{2}D gradient mode, namely multi-segmented shift gradients (MSG), was proposed for the first time here, and was demonstrated to provide improved metabolites separation, resulting in significant expansion of metabolic coverage.

2. Experimental

2-1. Reagents and materials

LC-MS grade ethanol, water, acetonitrile, and acetic acid were purchased from Merck KGaA (Darmstadt, Germany). The *G. glabra* sample was provided by L'Oréal (India).

2-2. Sample preparation

The grounded root material (1 g) was extracted 5 mL of ethanol/water (1:1, v/v) via ultrasonic agitation for 60 min. The resulting extract was left in darkness for 10 h, followed by filtration through 0.45 mm nylon filter membranes. The resulting extracts were diluted to a suitable concentration with ethanol/water (1:1, v/v) prior to injecting into the LC and LC×LC systems.

2-3. Analytical condition

Table 1 details the analytical conditions used in this analysis.

Comprehensive 2D-LC : Nexera-e

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Table 1 Analytical Conditions

Separation Conditions		
^{1}D column	:	Microbore cyano column (150 mmL.×1.0 mm I.D., 2.7 µm)
Mobile Phase A	:	0.1% acetic acid in H_2O
Mobile Phase B	:	0.1% acetic acid in ACN
Gradient	:	0-60 min, 35% B; 60 min, 80% B; 80 min, 100% B.
Flow-rate	:	10 µL/min
^{2}D column	:	Superficially porous C18 column (50×2.1 mm I.D., 2.7 µm).
^{2}D gradient	:	Illustrated in Fig.1.
Flow-rate	:	0.8 mL/min
Modulation	:	90 seconds
Detection		
Photo Diode Array Detector		
Wavelength range	:	210-400 nm
Acquisition frequency	:	12.5 Hz
Time constant	:	0.08 sec
Mass Spectrometry		
MS ionization mode	:	ESI positive and negative mode
Mass range	:	100-1000 m/z
Heat block temperature	:	250 °C
DL temperature	:	250 °C
Nebulizing gas flow	:	2 L/min
Interface voltage	:	3.5 kV
Detector voltage	:	1.8 kV
Software		
Shimadzu LabSolutions software ver. 5.65		
2D Software		
Chromsquare version 2.2.		
LC×LC-Assist software version 2.0		

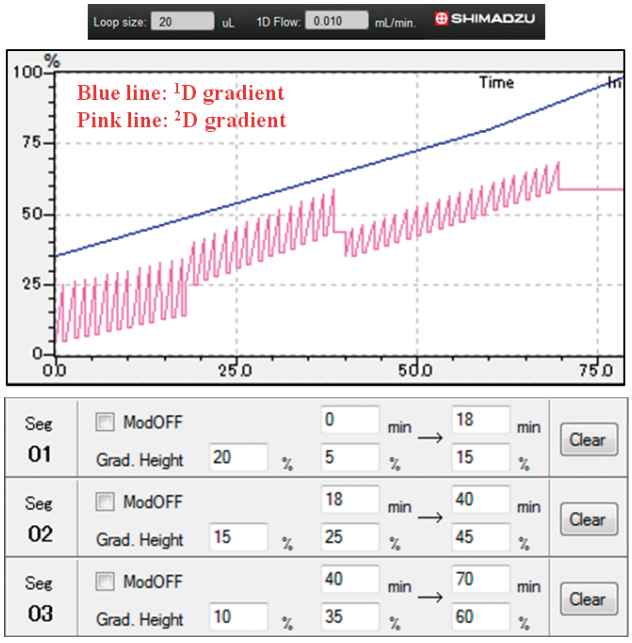

Fig. 1 Multi-segmented shift gradient program

3. Results and discussion

The analysis of *G. glabra* extract was first conducted using a LC-PDA-MS approach, employing the C18 column. Due to the high complexity of the sample, a considerable number of compounds overlapped (Fig.2), which is in agreement with earlier studies on licorice using a 1D-LC method.

Therefore, a 1D-LC separation technique is likely insufficient for a comprehensive analysis of a complex plant extract, and the use of multiple dimensions (i.e., separation and detection selectivity) is necessary. In this regard, the metabolic products of the *G. glabra* extract were subjected to RP-LC×RP-LC-PDA-MS analysis in order to identify the full suite of compounds in the "metabolic pool" of biosynthesized metabolites.

The coupling of RP-LC×RP-LC provides limited resolving power (i.e. peak capacity) due to the apparent similarity of the separation mechanisms in both dimensions when a conventional 2D full in-fraction (FIF) mode is employed. To improve separation of the mixture, a 2D shift gradient (SG) approach utilising a narrower range of organic solvent whilst gradually increasing the ACN proportion to create a progressively stronger ^{2}D gradient (as the ^{1}D gradient proceeded) was investigated and a total of three (multiple) different SG steps was employed (MSG). The contour plot for the RP-LC×RP-LC analysis of *G. glabra* extract under the MSG program is shown in Fig.3.

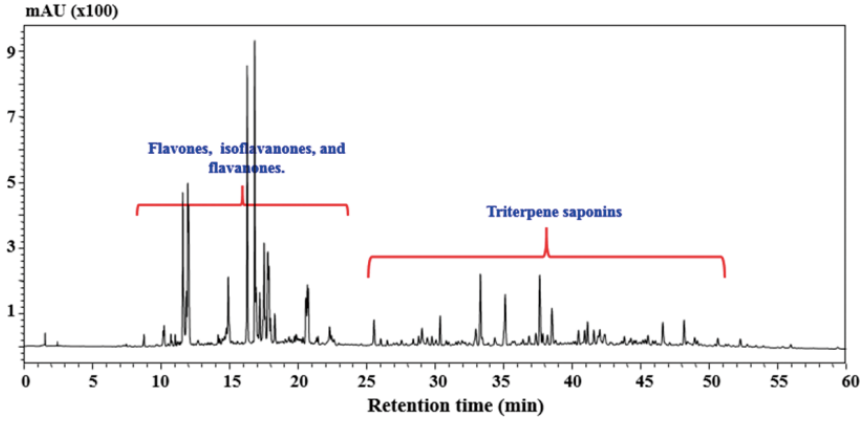

Fig. 2 1D-LC-PDA chromatogram of the licorice extract

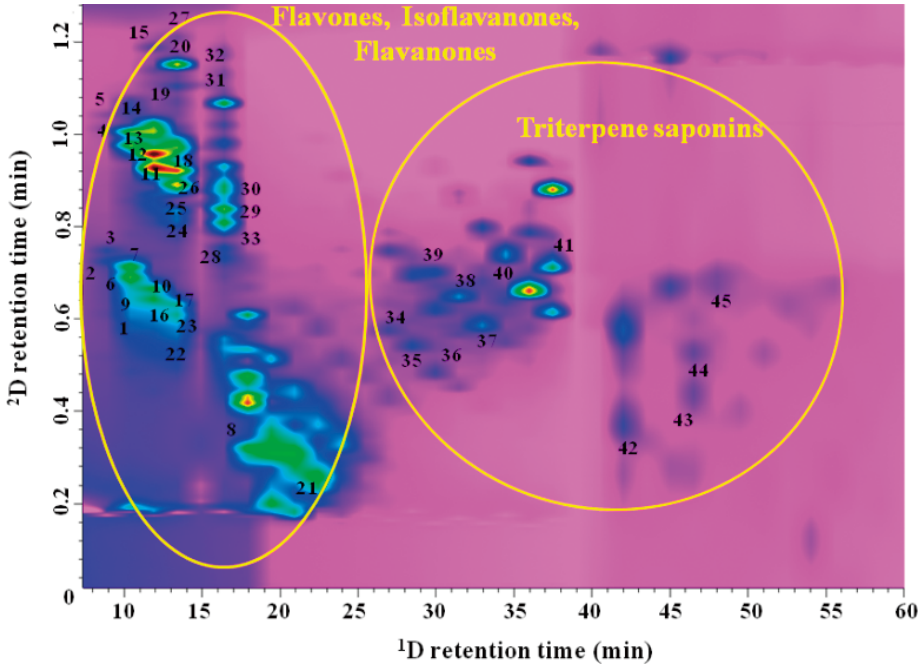

Fig. 3 RP-LC×RP-LC-PDA analysis of G. glabra extract using the multi-segmented shift gradient approach. The numbering of compounds is according to Table 2.

Table 2 Chemical components tentatively identified in *G. glabra* extract analysed using RP-LC×RP-LC-PDA/MS. Compounds are numbered in increasing retention order with reference to Fig.2.

No	$[M-H]^-$	λ_{max} (nm)	Identification
1	549.2	218, 274, 313	(Iso)liquiritin apioside
2	577.1	215, 275, 315	(Iso)violantin
3	577.1	215, 275, 315	(Iso)violantin
4	561.1	365	Glycyroside or isomer
5	561.1	365	Glycyroside or isomer
6	549.2	218, 274, 313	(Iso)liquiritin apioside
7	549.2	218, 274, 313	(Iso)liquiritin apioside
8	577.1	215, 275, 315	(Iso)violantin
9	577.1	215, 275, 315	(Iso)violantin
10	577.1	215, 275, 315	(Iso)violantin
11	725.2	365	Licorice glycoside A/C1/C2
12	725.2	365	Licorice glycoside A/C1/C2
13	725.1	365	Licorice glycoside A/C1/C2
14	711.2	283	Glucoliquiritin apioside or
15	725.2	365	Licorice glycoside A/C1/C2
16	577.1	215, 275, 315	(Iso)violantin
17	577.1	215, 275, 315	(Iso)violantin
18	725.2	365	Licorice glycoside A/C1/C2
19	921.2	370	NI
20	903.2	324	NI
21	549.1	273	NI
22	549.1	215, 273, 314	NI
23	577.1	215, 275, 315	(Iso)violantin
24	549.1	363	NI
25	725.2	365	Licorice glycoside A/C1/C2
26	695.1	370	Licorice glycoside B/D1/D2
27	737.2	310	NI
28	549.2	218, 274, 313	(Iso)liquiritin apioside
29	549.2	218, 274, 313	(Iso)liquiritin apioside
30	903.3	370	NI
31	725.2	365	Licorice glycoside A/C1/C2
32	903.2	321	NI
33	549.2	368	(Iso)liquiritin apioside
34	821.4	278	Uralsaponin B or isomers
35	821.4	278	Uralsaponin B or isomers
36	439.2	265, 309	Squasapogenol
37	441.2	284, 305	GP-B2
38	569.1	321, 298	12-Acetoxyganoderic acid F
39	337.1	286, 321	licochalcone A or isomers
40	367.1	288, 321	Glycycoumarin isomers
41	367.0	288, 321	Glycycoumarin isomers
42	819.3	328, 287	Licorice saponin E2
43	469.3	327, 289, 267	18β-Glycyrrhetinic acid
44	805.2	320	Licorice saponin C2
45	469.3	226, 281	Glycyrrhetic acid

A visual inspection of the 2D plot shows very promising peak spreading over the 2D separation space, with no evidence of distribution along the diagonal-line (which might suggest correlated retention), which might logically lead to overall improved compound coverage. The significant improvement of compound coverage can be justified by precise segmentation of the 2D gradient profiles into three different sections, with each segment having a narrow organic solvent composition range and with different gradient steepness.

By using RP-LC×RP-LC-PDA-MS set-up under MSG approach a total of ca. 120 compounds were detected. The major classes of compounds included flavanones, flavones, chalconoids, and triterpenes. Table 1 reports the tentative identification of the G. glabra constituents on the basis of their MS and diode array spectra data with reference to reported literature. The gain in compound coverage can be readily observed; 1D-LC analysis (Fig.2) enabled ca. 66 compounds to be detected, in comparison to RP-LC×RP-LC analysis indicating detection of ca. 120 compounds (Fig.3), corresponding to a ca. two-fold increase in number of detected components. The contour plot demonstrates that the compounds are organised mainly into two major structural clusters in the 2D separation space. The justification for this structural feature is the organised variation in chemical structure of molecules of a particular class that leads to minor changes in the relative affinity with the stationary phases, in which the ^{2}D gradient plays a major role in eluting the components at different solvent strength, leading to small differences in retention position in ^{2}D.

4. Conclusions

In conclusion the prospects of employing MSG to expand the metabolic coverage in RP-LC×RP-LC-PDA-MS analysis of *G. glabra* extract are demonstrated. A combination of microbore cyano (^{1}D) and superficially porous octadecylsilica (^{2}D) phases provided a broad overview on the metabolite composition of *G. glabra* extract, with a focus on the flavones, isoflavanones and triterpene saponins region, and also on differentiation of isobaric components using two separation mechanisms. Both orthogonality and metabolic coverage are significantly enhanced through applying MSG for the ^{2}D separation stage, allowing deeper characterization of the metabolic composition of *G. glabra* extract. The described MSG approach can be adapted to high resolution RP-LC×RP-LC metabolic analyses of other complex plant derived extracts.

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