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Identification of *Scutellaria Baicalensis* in Traditional Chinese Medicine Preparations by LC/MS/MS Fingerprinting Method

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ABSTRACT

A high-performance liquid chromatography coupled with electrospray ionization (ESI) tandem mass spectrometry (LC/MS/MS) method was developed for the chemical fingerprint analysis of *Scutellaria baicalensis* and rapid identification of major compounds in the fingerprints. The chromatography was carried on a C18 analytical column (150 mm x 4.6 mm, 2.7 μm) with gradient elution using acetonitrile and 0.25% (v/v) formic acid. Mass spectrometry was performed in the negative ion mode using ESI. Sixteen main peaks in the fingerprints were identified by comparing the UV and MS spectra data with those from the authentic standards and literature. This method was successfully employed for the identification of Scutellariae Radix in three traditional Chinese medicine preparations, namely Shin Yi Ching Fey Tang, Huang Lien Chieh Tu Tang and Lung Tan Hsieh Kan Tang. The developed fingerprint assay was specific and could be readily utilized for comprehensive evaluation of Scutellariae Radix.

Key words: LC/MS/MS, fingerprint, *Scutellaria baicalensis*, multiple reaction monitoring (MRM)

INTRODUCTION

The fingerprint of a herbal extract can be defined as a chromatographic pattern of common pharmacologically active and chemically characteristic components. The entire pattern of compounds can be applied to determine not only the presence or absence of desired markers or activities, but the complete set of ratios of all detectable analytes. Thus, fingerprint analysis of traditional Chinese medicines (TCMs) represents a qualitative approach to determine a variety of components for the purpose of ensuring the quality and stability of TCM preparations (TCMPs)[1-5].

Scutellaria baicalensis Georgi (Huang-qin in Chinese) is one of the most widely used TCM and is officially listed in the Chinese Pharmacopoeia. Its roots have been used for its anti-inflammatory and anti-cancer properties, treating bacterial and viral infections of the respiratory and gastrointestinal tracts, cleaning away heat, moistening aridity, purging fire, detoxifying toxicosis, reducing total cholesterol level and decreasing blood pressure. This herb also possesses cholagogic, diuretic and cathartic actions. TCMPs that contain Scutellariae Radix as a major ingredient are widely used in oriental countries[6-10].

Scutellariae Radix contains a variety of sterols, phenylethanoids, flavones, amino acids and essential oils. Its dried roots contain over 30 kinds of flavonoids, such as wogonin, baicalin, wogonoside, baicalein, wogonin 7-*O*-glucuronide, oroxylin A and oroxylin A 7-O-glucuronide. Baicalin, baicalein, wogonin and oroxylin A are the main active components in Scutellariae Radix[11-14]. Baicalin is the most abundant component and has anti-allergic[15], anti-inflammatory[16], anti-HIV[17], anti-tumor[19-21], anti-oxidant and free radical scavenging[22-23] and anti-SARS coronavirus effects[24]. Baicalein possesses anti-HIV[17-18], anti-tumor[21], anti-oxidant and free radical scavenging effects[22-23]. Wogonin has anti-respiratory syncytial virus[25], anti-hepatitis B virus, anti-tumor[21], anti-oxidant and free radical scavenging effects[23]. Oroxylin A has anti-respiratory syncytial virus activity[25].

Fingerprint analysis is a viable means of comprehensive analysis for TCM and TCMP. At present, the use of HPLC/UV to establish the fingerprint spectra of TCM is widely accepted method. However, this method failed to detect components that are not UV-active[26-27].

HPLC coupled with diode array detection (DAD) and MS spectral methods have proven to be a powerful tool for the rapid characterization of components in natural products. DAD and MS are highly sensitive detectors and can provide abundant structural information, which facilitate the structural identification of unknown compounds[28-31].

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In this paper, LC/MS/MS was employed to establish the fingerprint of Scutellariae Radix herbal extract. The method was then applied for the identification of Scutellariae Radix in three traditional Chinese medicine preparations, Shin Yi Ching Fey Tang, Huang Lien Chieh Tu Tang and Lung Tan Hsieh Kan Tang.

MATERIALS AND METHODS

I. *Standard Solution Preparation*

The reference substances (baicalin, wogonoside, baicalein, wogonin and chrysin) were accurately weighed and dissolved in 70% methanol to obtain solutions of suitable concentration. The standard solutions were stored under refrigeration at 4°C when not in use. The reference substances (> 98% purity) were purchased from Nacalai.

II. *Sample Collection and Preparation*

Fifty-two samples of the Scutellariae Radix herb were obtained from Taiwan market. Their authentic origin, *Scutellariae baicalensis* Georgi, was confirmed by their morphological characteristics, microscopic examination and polymerase chain reaction (PCR) analysis[32].

Each powdered sample (0.2 g) was extracted with 70% methanol (10 mL) by ultrasonication at room temperature for 30 min. The extract was filtered with a 0.45 μm Millipore filter membrane. The filtrate was then used for LC/MS/MS analysis.

Five batches of Shin Yi Ching Fey Tang, Huang Lien Chieh Tu Tang and Lung Tan Hsieh Kan Tang, each equivalent to 0.2 g of Scutellariae Radix, were extracted with 70% methanol (10 mL) by ultrasonication at room temperature for 30 min. The extracts were filtered with a 0.45 μm Millipore filter membrane. The filtrates were then used for LC/MS/MS analysis.

III. *LC/MS/MS Analysis*

A Waters 2695 Separations Module HPLC system (Waters Corp., Milford, MA, USA) consisting of a quaternary solvent delivery system, an on-line degasser, an autosampler and a DAD was used for acquiring chromatograms and UV spectra. Chromatographic separation was performed over a HALO C18 analytical column (150 × 4.6 mm, 2.7 μm) (Advanced Materials Technology, USA). The mobile phase consisted of acetonitrile (A) and 0.25% (v/v) formic acid (B) with a gradient elution program as follows: 20 - 27% (A) in 0 - 18 min, 27 - 45% (A) in 18 - 28 min, 45 - 50% (A) in 28 - 33 min and 50 - 20% (A) in 33 - 35 min. A re-equilibration time of 11 min was used between HPLC runs. The flow rate was set at 0.5 mL/min and the sample injection volume at 10 μL. With the use of solvent splitting, 0.25 mL/min of the column effluent was delivered to the ion source of the mass spectrometer. DAD spectra recorded from 190 to 400 nm was used for peak characterization and the detection wavelength was set at 270 nm for fingerprint analysis.

Mass spectra were obtained on a Waters 2695 Separations Module HPLC system coupled to a Micromass Quattro Premier triple-quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray source and Masslynx version 4.1 software. Scutellariae Radix was analyzed using electrospray ionization in the negative ion mode. The MS/MS conditions were as follows: capillary voltage, 3.0 kV; cone voltage, 100V; source temperature, 120°C, desolvation gas temperature, 350°C; desolvation gas flow, 674 L/h; cone gas flow, 71 L/h nitrogen. The argon collision gas pressure was adjusted to 3.46×10^{-3} mbar. The collision energy was varied and optimized for each MRM transition. The transitions monitored were *m/z* 919.4 > 459.4 at 20 eV, 592.4 > 365.4 at 50 eV, 547.4 > 337.4 at 32 eV, 459.4 > 283.4 at 15 eV, 373.4 > 343.4 at 23 eV, 283.4 > 268.4 at 15 eV, 269.4 > 167.4 at 32 eV and 253.4 > 143.4 at 35 eV.

RESULTS AND DISCUSSION

I. *Optimization of Extraction Procedure and Chromatographic Conditions*

Experimental variables, such as the extraction method and chromatographic conditions, were optimized in order to obtain a complete chromatographic fingerprint of Scutellariae Radix. Different extraction solvents, including water, 70% methanol, methanol and ethanol, were evaluated based on the total number of characteristic peaks obtained. The results indicated that 70% methanol was the most suitable extraction solvent. As TCMPs are generally extracted with water, we compared the HPLC-UV chromatograms of Scutellariae Radix and Shin Yi Ching Fey Tang after extracting them separately with water and 70% methanol. There was no significant difference in the chromatograms, indicating that both solvents were suitable for the extraction of Scutellariae Radix in TCMP. Based on the results obtained, 70% methanol was chosen as the extraction solvent for the entire analysis.

When the DAD detection wavelength was set in the range of 190 to 400 nm, it was observed that more peaks of interest and a smoother baseline could be obtained at a wavelength of 270 nm. Therefore, 270 nm was chosen as the optimum detection wavelength for the analysis. Different mobile phase compositions, including acetonitrile, methanol and water were also tested. Formic acid was added to the mobile phase to enhance the peak resolution and eliminate peak tailing. From the testing, a mobile phase system consisting of acetonitrile and water containing 0.25% (v/v) formic acid in gradient elution mode was chosen for the analysis.

MS scans of Scutellariae Radix in negative and positive ion modes showed different sensitivity. The scan in negative ion mode provided more information than that in the positive ion mode (Figure 1). Therefore, the negative ion mode was used for the analysis.

II. *Identification of Major Components in Scutellariae Radix*

The chemical constituents of Scutellariae Radix were determined by LC/MS/MS. Peaks 5, 9, 11, 13 and 14 in the MS chromatogram were unequivocally identified as baicalin, wogonoside, baicalein, wogonin and chrysin by matching the UV and MS spectra with those of the reference compounds. Owing to the lack of analytical standards, the other 11 peaks could only be tentatively assigned by comparing their UV spectra and MS data with those reported in previous studies. The details of the identified components were summarized in Figure 2 and Table 1.

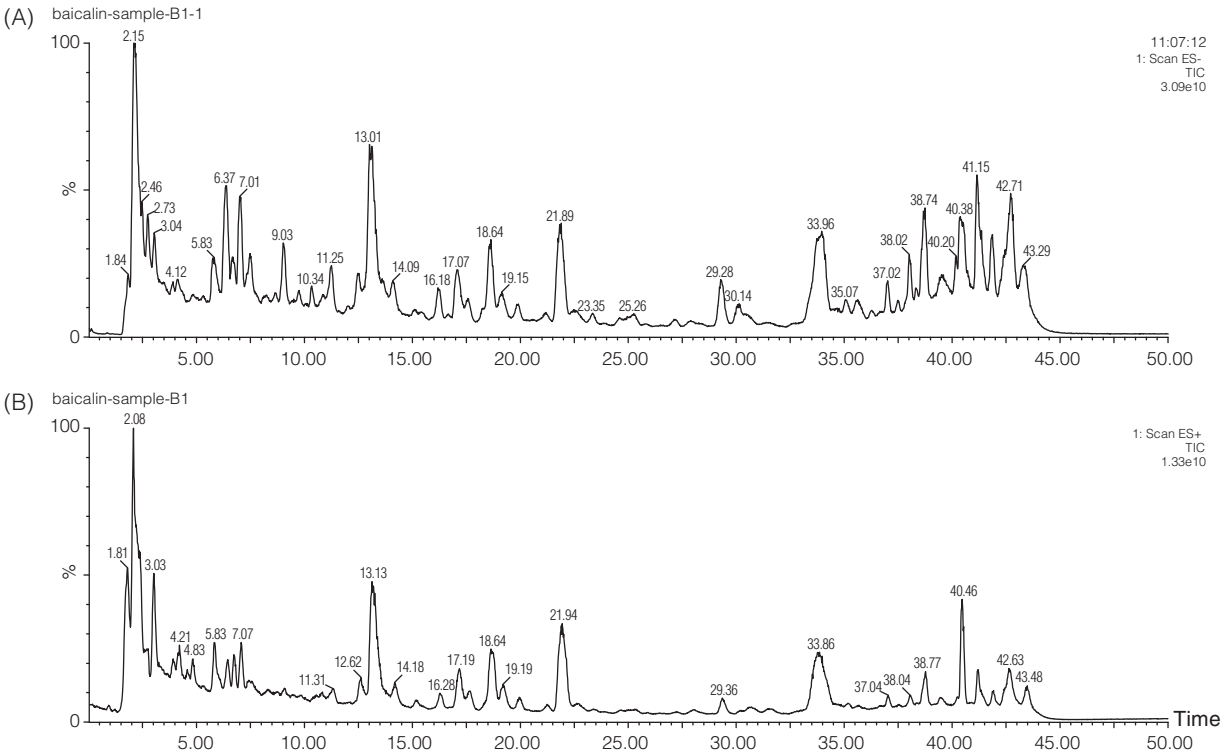

Figure 1. MS scans of Scutellariae Radix in different modes, (A) negative mode and (B) positive mode.

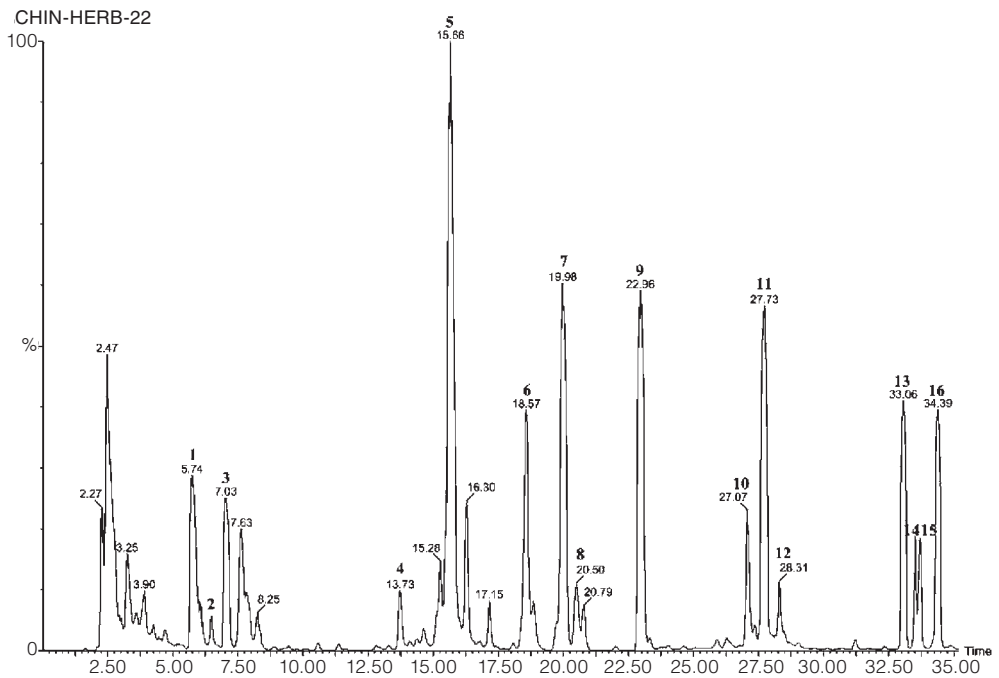

Figure 2. Compounds in *Scutellariae Radix* (negative ion mode).

Table 1. Compounds identified in *Scutellariae Radix* by HPLC-DAD-MS-MS

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$(m/z)	MS^2(m/z)	Identification
1	5.74	272, 313	547	510,487,457,427,367,337	Chrysin -6-*C*-arabinose-8-C-glucose
2	6.48	290, 330	623	461	Isorhamnetin-7-*O*-rhamnosyl-glucoside
3	7.03	272, 314	547	510,487,457,427,367,337	Chrysin -6-*C*-glucose-8-*C*-arabinose
4	13.73	268, 334	951	574,497,475,299	5,2'-Dihydroxy-6'-methoxy-7-*O*-glucuronide
5*	15.66	276, 314	445	269	Baicalin
6	18.57	278	891	445, 269	Galengin-7-*O*- glucuronide
7	19.97	271, 311	919	459, 283, 268	Oroxylin A-7-*O*-β-D-glucuronide
8	20.50	266, 304	429	253, 175	Chrysin-7-*O*-β-D-glucuronide
9*	22.96	273	459	283, 268, 175	Wogonoside
10	27.07	279, 322	269	251, 241	Norwogonin
11*	27.72	275,321	269	251, 241	Baicalein
12	28.31	268,333	299	284	5,7,4-Trihydroxy-8-methoxy flavone
13*	33.06	274	283	268	Wogonin
14*	33.51	269,315	253	143	Chrysin
15	33.69	268	373	358, 343	Skullcapflavon II
16	34.39	270, 317	283	268	Oroxylin A

* Identified by comparing experiment data with those of standard compounds

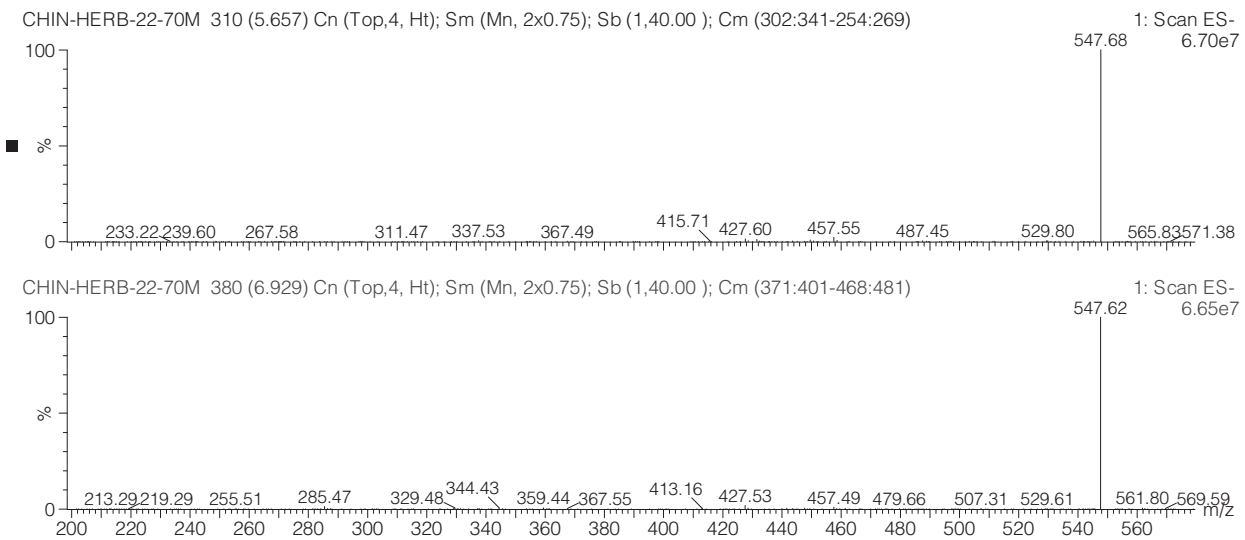

Figure 3. MS spectra of peak 1 (top) and peak 3 (bottom).

Parent ion masses (*m/z* 445.4, 459.4, 269.4, 283.4 and 253.4) of the marker components, baicalin, wogonoside, baicalein, wogonin and chrysin were obtained from the LC/MS/MS analyses of the reference substances. These data were written into the LC/MS/MS fingerprint database for library search. Together with retention time information obtained from HPLC, these marker components could be easily identified using the LC/MS/MS fingerprint database.

In Figure 3, peaks 1 and 3 are isomers with a molecular weight of 548 Da. To further investigate the structures of the two isomers, we employed tandem mass spectrometry on the $[M-H]^-$ ion at *m/z* 547 separately, with a cone voltage of 100V and collision energy of 32eV. Based on the ESI-MS^2 spectral data of peaks 1 and 3, they were found to have similar spectra (Figure 3). In the ESI-MS^2 spectra, ions of $[M-H-60]^-$, $[M-H-90]^-$ and $[M-H-120]^-$ were observed and considered as

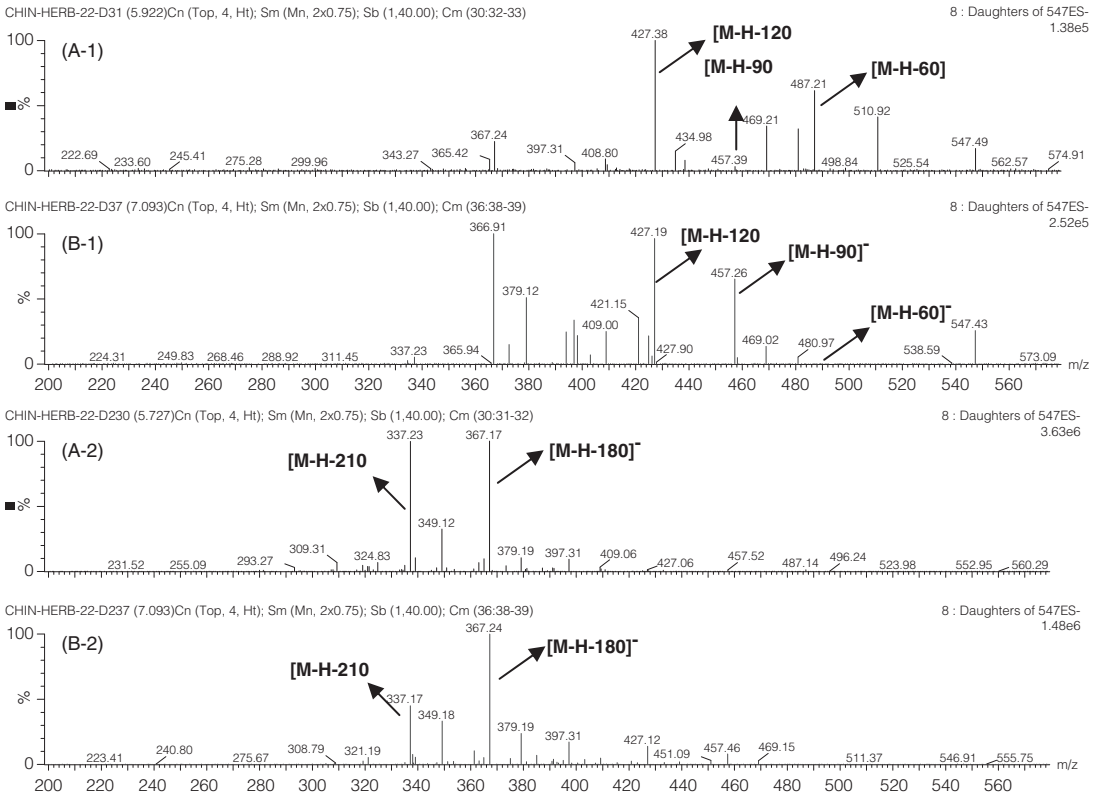

Figure 4. ESI-MS^2 spectra of peak 1 (A-1 and A-2) and peak 3 (B-1 and B-2). The collision energy used in A-1 and B-1 was 25 eV, while that in A-2 and B-2 was 32 eV.

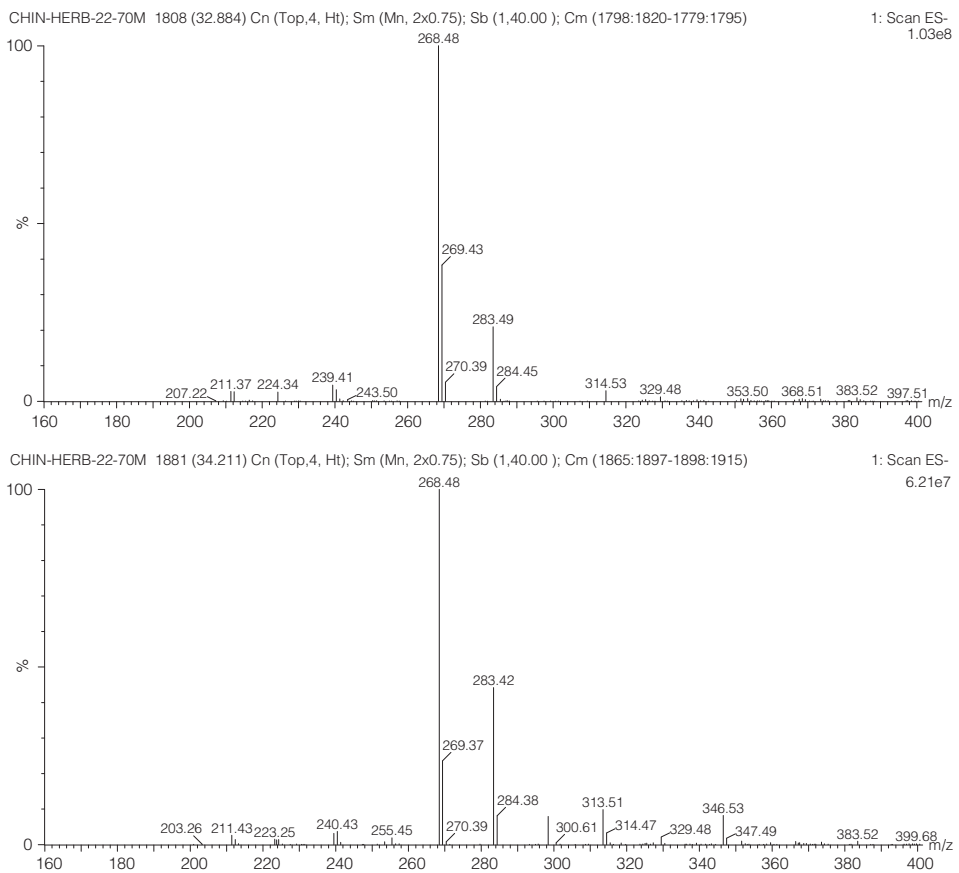

Figure 5. MS spectra of peak 13 (top) and peak 16 (bottom).

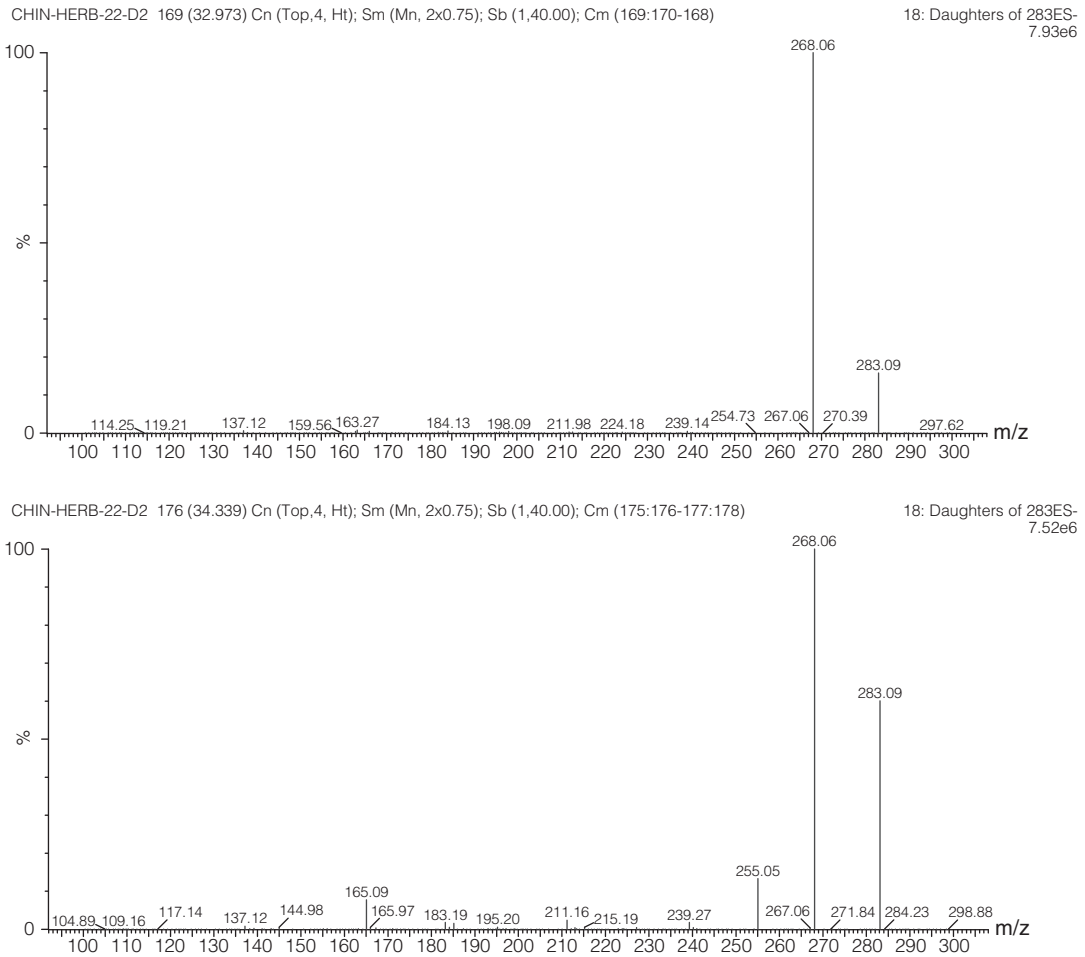

Figure 6. ESI-MS^2 spectra of peak 13 (top) and peak 16 (bottom).

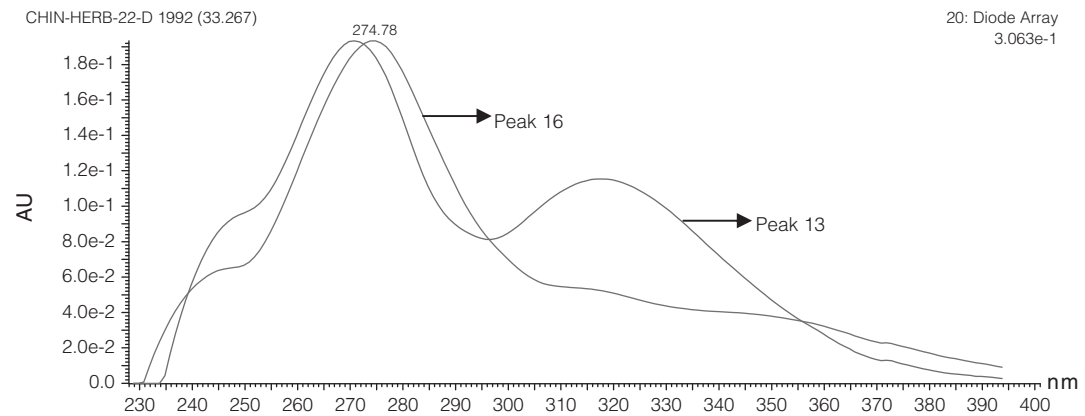

Figure 7. UV spectra of peaks 13 and 16.

characteristic ions of *C*-glycosidic flavonoids. Ions with *m/z* 367 [*M*-H-180]$^-$ and 337 [*M*-H-210]$^-$ suggested the presence of two glycosyl groups. In previous literature[33], ions at *m/z* 487 [*M*-H-60]$^-$ and *m/z* 427 [*M*-H-120]$^-$ could be produced by the cleavage of 0.3 band of *C*-pentosyl and 0.2 band of *C*-hexosyl, respectively, and the sugar substitution at C-6 position could offer the most intense fragments. According to the relative abundance of *m/z* 487 and *m/z* 427, peaks 1 and 3 were thus identified as Chrysin-6-*C*-arabinose-8-*C*-glucose and Chrysin-6-*C*-glucose-8-*C*-arabinose, respectively. In

addition, it was observed that *m/z* 367 [*M*-H-180]$^-$ and *m/z* 337 [*M*-H-210]$^-$ correlated with *m/z* 427 [*M*-H-120]$^-$ and *m/z* 487 [*M*-H-60]$^-$ (Figure 4).

The mass spectra of peaks 13 and 16 exhibited the molecular ion of *m/z* 283. Their ESI-MS^2 spectral data were found to be similar (Figure 5). The ESI-MS^2 spectrum of the [*M*-H]$^-$ ion at *m/z* 283 exhibited the methoxylated flavone characteristic loss of CH_3 (15 Da) (Figure 6), resulting in an ion at *m/z* 268. Peaks 13 and 16 could not be identified from their MS and ESI-MS^2 spectra. From their UV spectra (Figure 7), they were identified as wogonin and oroxylin A, respectively.

In terms of water solubility, peaks 14 was more polar than peak 16. In general, compounds coupled with glucose exhibited higher solubility in water, but as glucuronic acid is more polar than glucuronide, peaks 7 and 8 were identified as oroxylin A-7-*O*-β-D-glucuronide and chrysin-7-*O*-β-D-glucuronide, respectively.

III. *Reproducibility of the LC/MS/MS Method*

The daughter ion screening method established was used for the analysis of three TCMPs, namely, Shin Yi Ching Fey Tang, Huang Lien Chieh Tu Tang and Lung Tan Hsieh Kan Tang. The marker components, baicalin, wogonoside, baicalein, wogonin and chrysin in the TCMPs could be identified useing the LC/MS/MS fingerprint database (Figure 8 - 11).

The MRM spectra of the marker components and three TCMPs (Figure 12 - 15) were also found to be similar to that of Scutellariae Radix, indicating the feasibility of this method for the identification of Scutellariae Radix in TCMPs (Figure 16). The *m/z* value 592 > 365 in the MRM spectra was not listed in the Table 1, as the compound of molecular weight of 592 Da was not identified.

CONCLUSIONS

A simple, fast, stable and accurate LC/MS/MS method was developed for the fingerprint analysis of Scutellariae Radix from 52 samples. The marker components of Scutellariae Radix could be identified and the resolution factors of most of the characteristic peaks were excellent. The method was successfully used for the identification of Scutellariae Radix in 3 TCMPs, Shin Yi Ching Fey Tang, Huang Lien Chieh Tu Tang and Lung Tan Hsieh Kan Tang. The results suggested that fingerprint analysis by LC/MS/MS is a powerful tool for the identification of Scutellariae Radix in TCMPs.

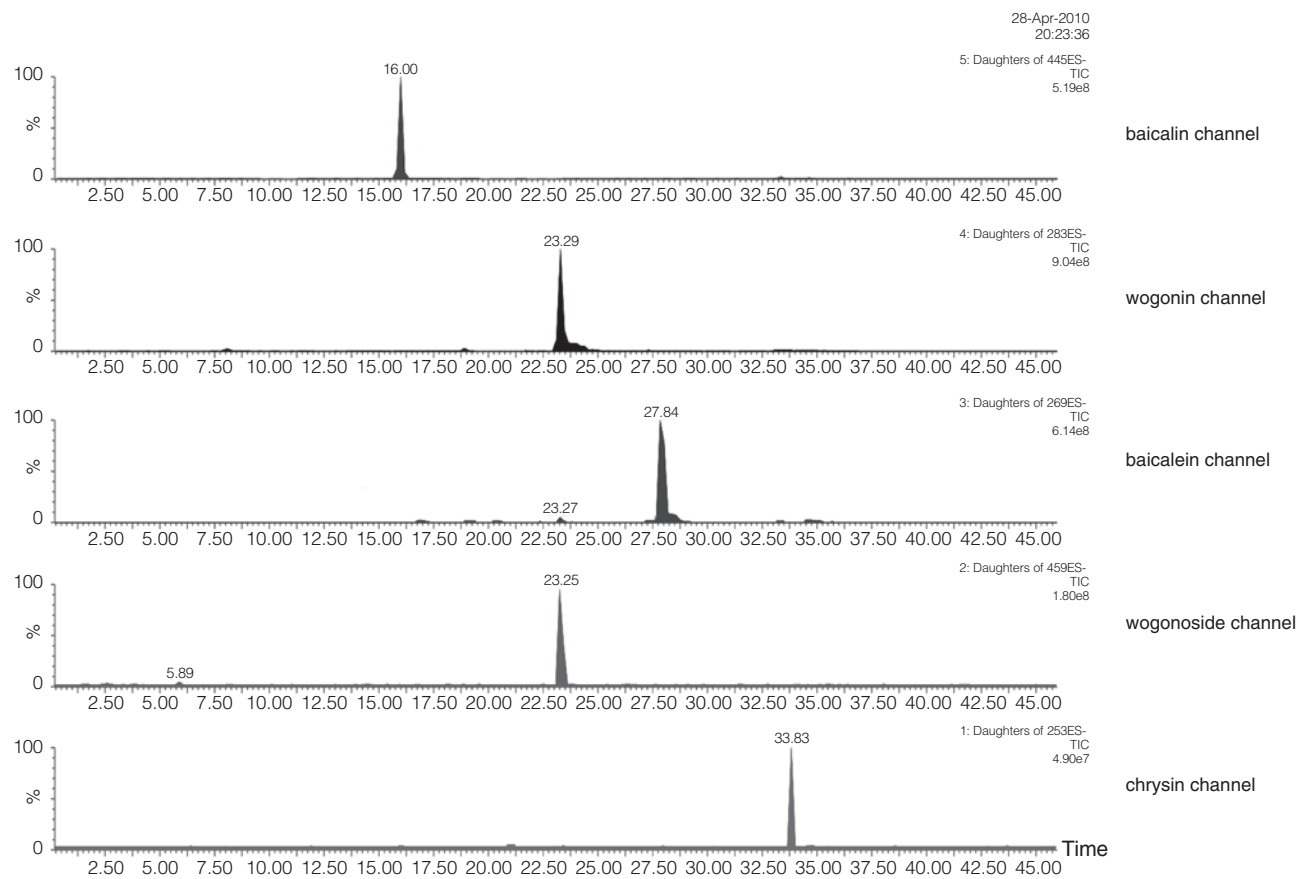

Figure 8. Daughter ion scans of the reference substances.

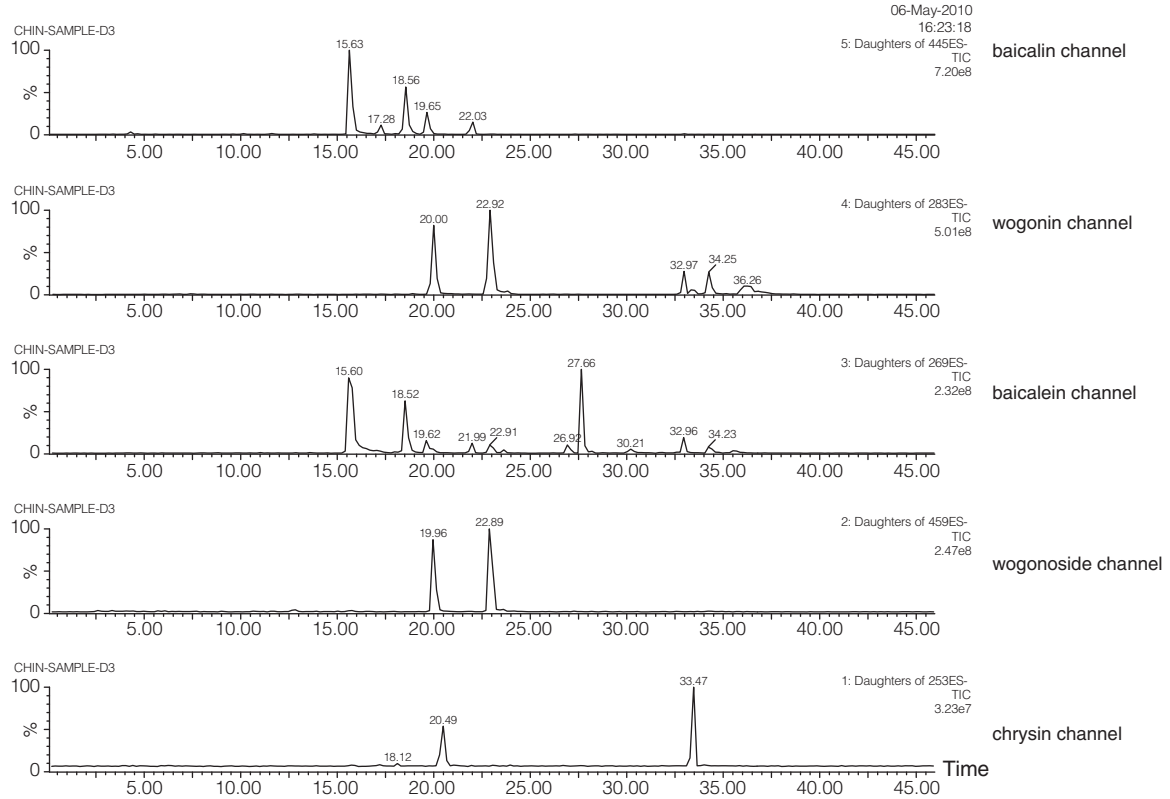

Figure 9. Daughter ion scan of Shin Yi Ching Fey Tang.

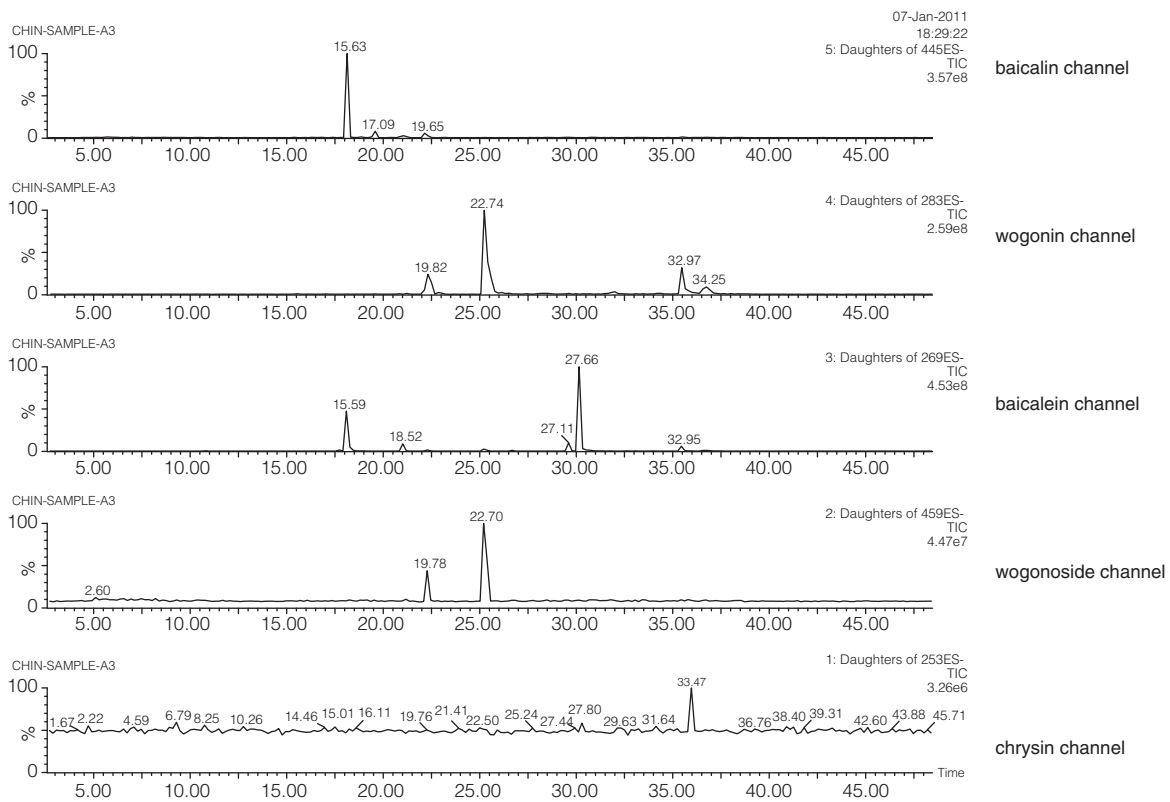

Figure 10. Daughter ion scan of Huang Lien Chieh Tu Tang.

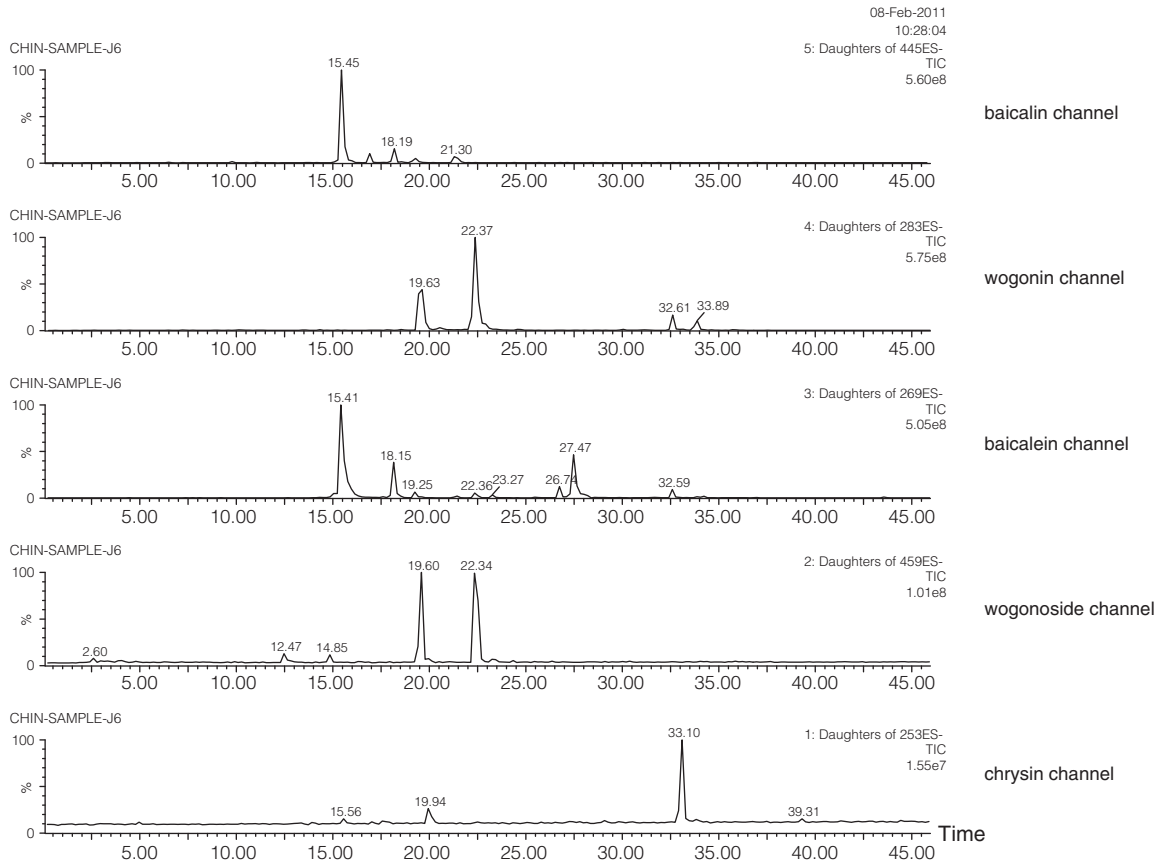

Figure 11. Daughter ion scan of Lung Tan Hsieh Kan Tang.

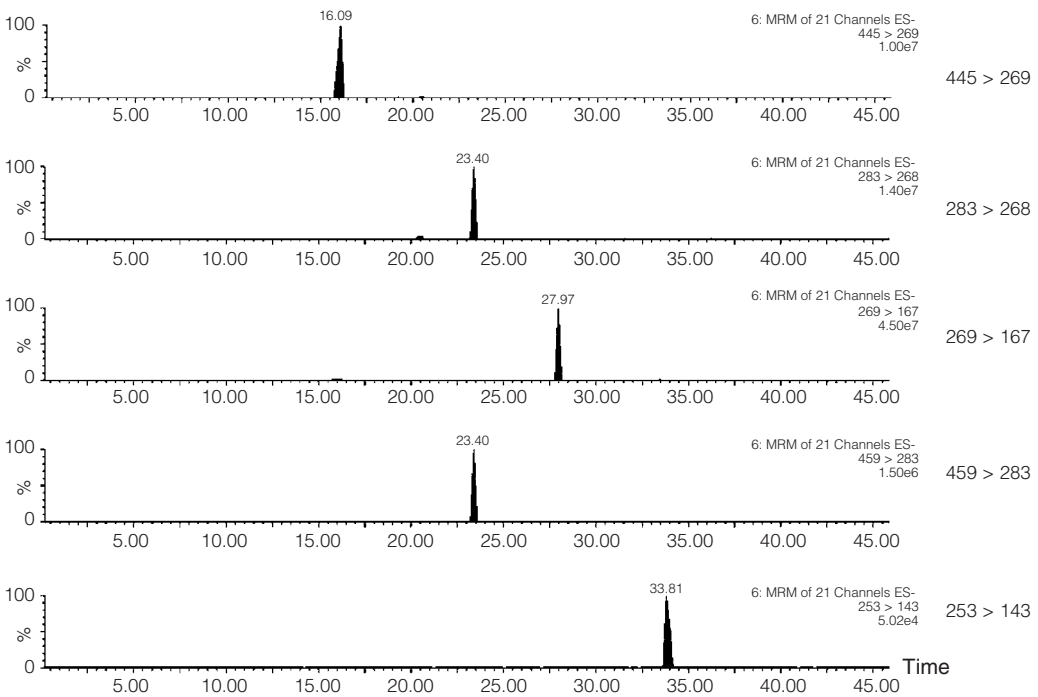

Figure 12. MRM ion scans of the reference substances.

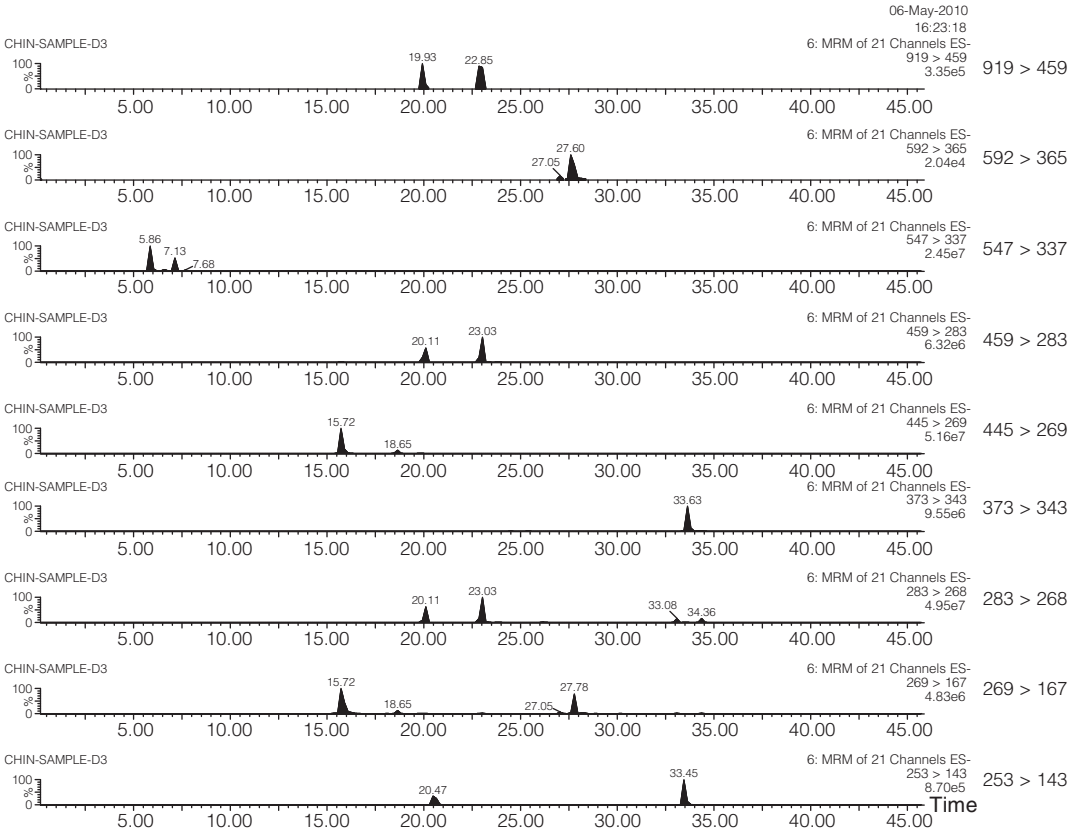

Figure 13. MRM ion scan of Shin Yi Ching Fey Tang.

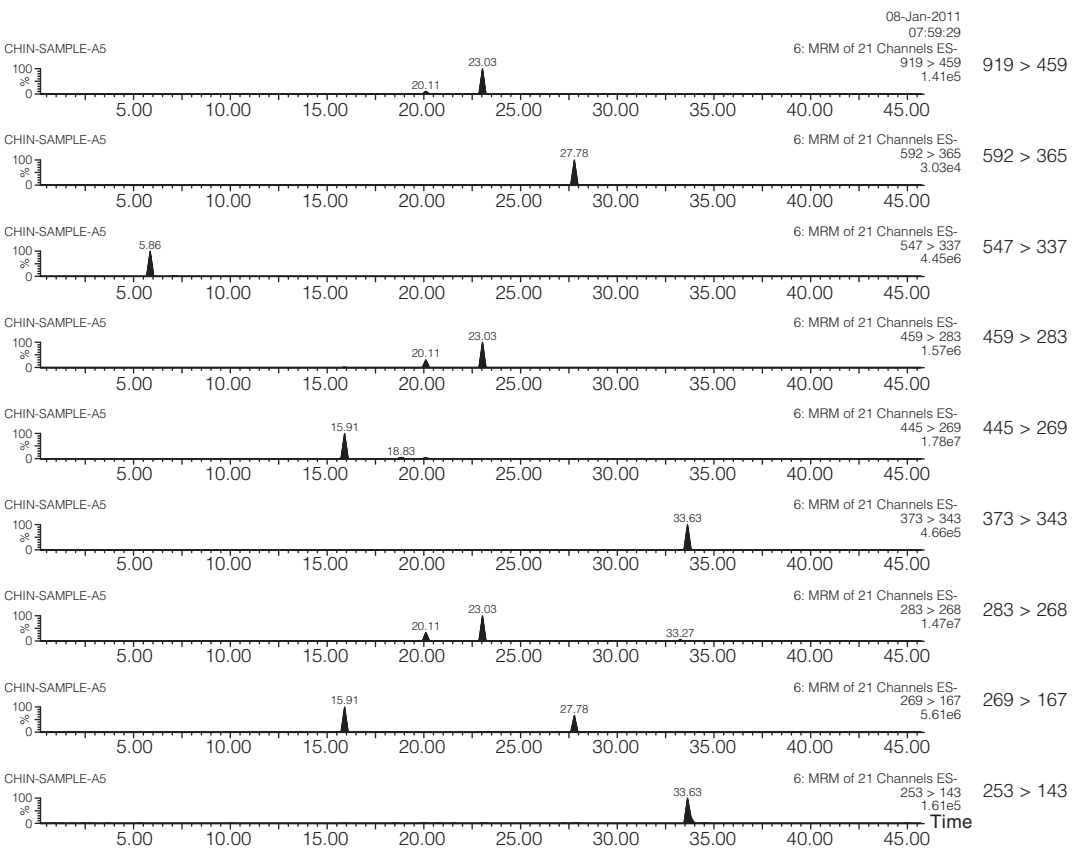

Figure 14. MRM ion scan of Huang Lien Chieh Tu Tang.

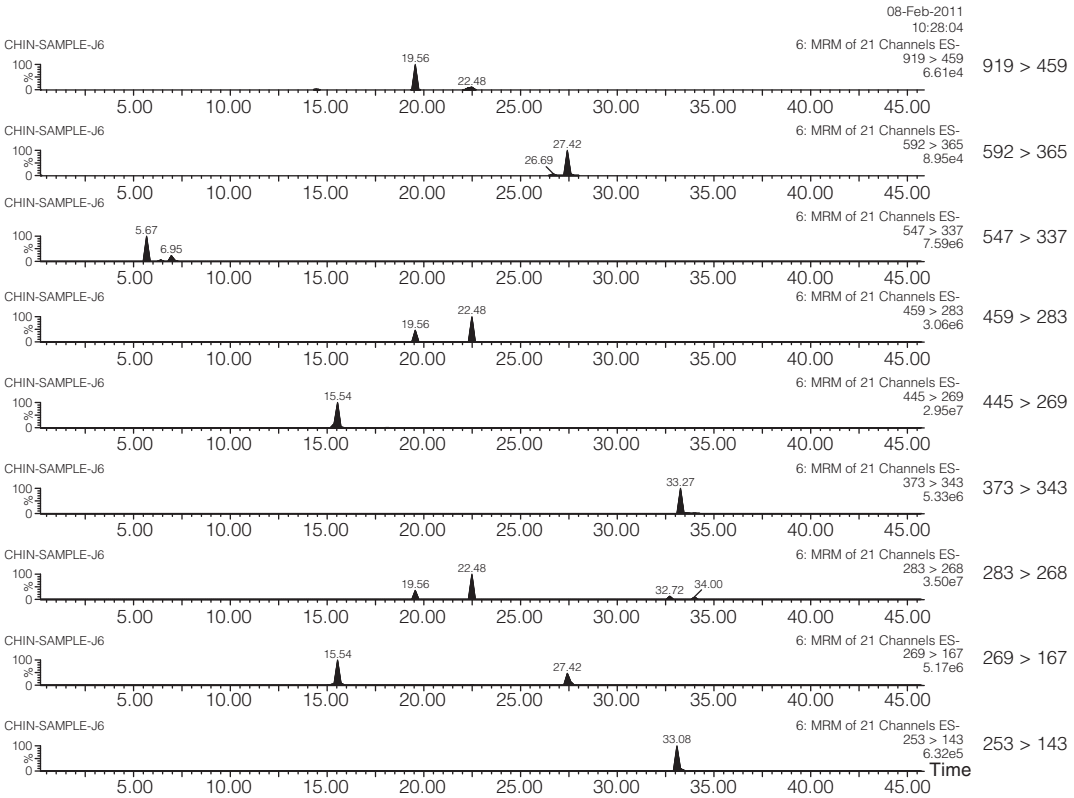

Figure 15. MRM ion scan of Lung Tan Hsieh Kan Tang.

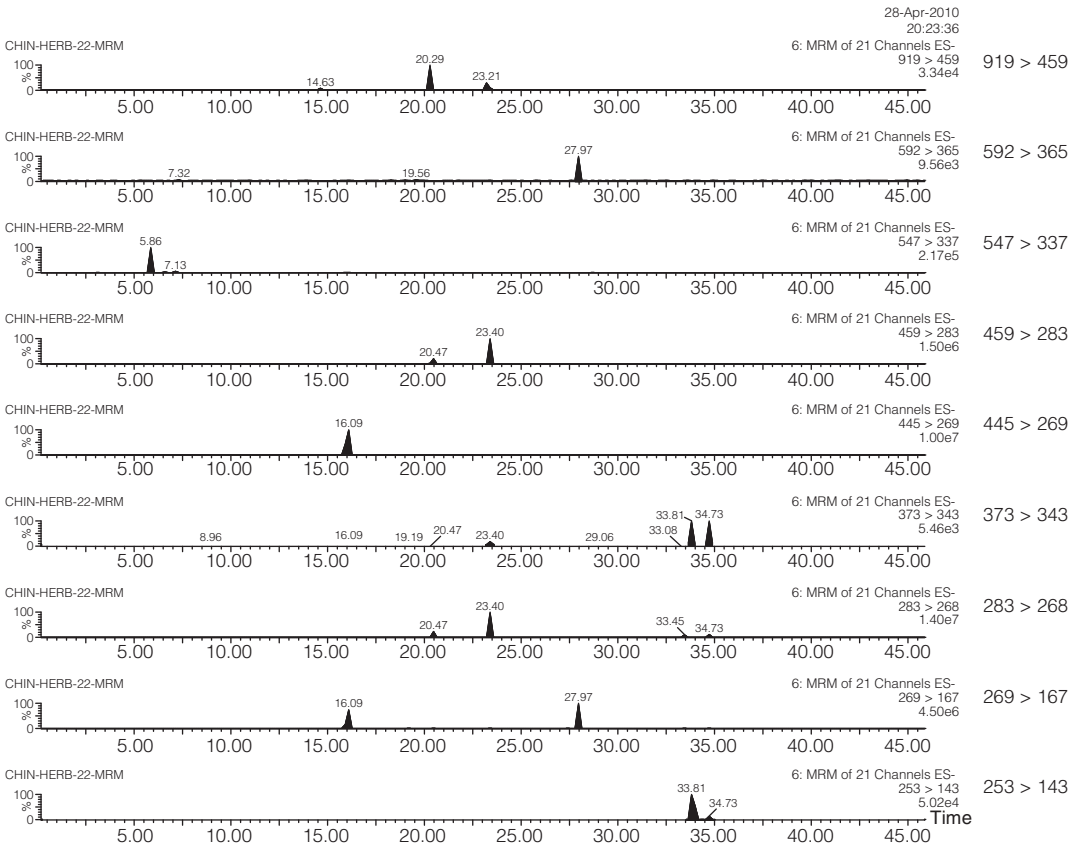

Figure 16. MRM ion scan of Scutellariae Radix.

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