LC-MS based metabolite fingerprinting and profiling of a traditional Chinese medicine YANG XIN® formulation

By

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Abstract

For thousands of years, Traditional Chinese Medicine (TCM) has been used to treat or prevent various diseases, for this reason western countries have been attracted to and use TCM together with allopathic medicine. YANG XIN® is a TCM formulation used for nervous fatigue and consists of a 500 mg proprietary blend of concentrated extracts from 18 plants. In this study, liquid chromatography coupled to a quadruple time of flight (q-TOF) mass spectrometer was used for identification of constituents and quantification of chemical markers for quality control of the YANG XIN® formulation.

Chapter One is an overview of TCM in general and YANG XIN® formulation in particular and its composition and usage. A brief review on the definition of chronic fatigue syndrome (CFS) and its symptoms are also included.

Chapter Two talks about metabolite fingerprinting and profiling of YANG XIN® formulation by using LC-MS and NMR. It also describes the process from sample extraction to sample preparation and analysis, in addition to the identification of the analytical markers and the strategy used for selection of analytical markers in plant ingredients.

Chapter Three describes the quantification process of YANG XIN® markers starting with sample preparation and ending with method validation.

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I dedicate this thesis to my mother, Nuhair Abdulrahman Surrati, my father, Jarallah Sa'eed Almalki, my brother, Malik Jarallah Almalki for their continuous support and their endless love and care; without them I could not achieve what I achieve today.

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List of Abbreviations

®	Registered trademark
°C	Degree centigrade
µL	Microliter
µM	Micromolar
^{1}H NMR	Proton nuclear magnetic resonance
CE	Capillary electrophoresis
CFS	Chronic fatigue syndrome
DBE	Double bond equivalent
DMSO	Dimethyl sulfoxide
EIC	Extracted ion chromatogram
EMA	European Medicines Agency
ESI	Electrospray ionization
FDA	Food and Drug Administration
g	Gram
GC	Gas chromatography
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
mL	Milliliter
mm	Millimeter

MS	Mass spectrometry
NMR	Nuclear magnetic resonance
Q-TOF	Quadruple time-of-flight
TCM	Traditional Chinese Medicine
TIC	Total ion chromatogram
TLC	Thin layer chromatography
WHO	World Health Organization

Chapter 1: Literature review

1.1 Introduction

For thousands of years, Traditional Chinese Medicine (TCM) has been used to treat or prevent various diseases; for this reason western countries have been attracted to and use TCM together with allopathic medicine [1]. However, the quality of TCM products are usually influenced by their different plant species, and their corresponding chemical composition, harvest season, storage, and geographic origins [1].

TCM formulations are usually made of multiple herbs; consequently, in order to standardize each formulations to help manufacturers to have consistent products, a so-called marker compound is selected for each plant included in the formulation. There are two types of marker compounds, the first is an active constituent marker, which is a compound or group of compounds that contributes to therapeutic action, and the second is an analytical marker, which is a compound or a group of compounds that does not contribute to the therapeutic action but serve only for analytical purposes which was used in this study. If we used the marker for the purpose of quality control study only, then a statement which include the content of the marker should not be included on the product label and this information should be available on request [2].

Analysis of such complicated mixtures like TCM formulations bears substantial challenges [3]. Therefore, identification of chemical constituents and quantification of selected chemical markers for quality control of TCM products are important to standardize herbal medicine to ensure efficacy and safety [4].

Chemical fingerprinting is one of the techniques used to ensure quality control of herbal preparations and to distinguish the herbal preparations from other related species [5].

Chronic fatigue syndrome (CFS) is a complex disease defined as: clinically unexplained, persistent or relapsing fatigue of at least 6 months duration and concurrent occurrence of at least 4 accompanying symptoms such as significant impairment of memory and muscle pain [6]. The symptoms of chronic fatigue syndrome can be classified into two groups: somatic and psychological symptoms [6]. Nervous fatigue is one of the psychological symptoms of CFS including sleep disorders with periodic movement disorder, excessive daytime sleepiness, apnea and narcolepsy, impaired short-term memory, headaches, tinnitus, anxiety, and depression. Currently a definitive treatment to cure nervous fatigue is not available. Since medical treatment of CFS is used to relieve symptoms [6], it is not surprising that utilization of TCM has been common in CFS. Eleven TCM formulations have been reported for the treatment of CFS [6]. For none of them except Yi-Gan San has, a quality control study been done [7,8]. YANG XIN® formulation is also used to treat nervous fatigue and it is the object of this study. This formulation consists of 500 mg of a proprietary blend of concentrated extract of 18 plants.

1.2 Project rationale

To the best of our knowledge, there is no report on the development of methods for the quality control of YANG XIN® formulation. Consequently, the use of liquid chromatography - mass spectrometry (LC-MS) is a well-accepted analytical technique for the identification of a potential analytical marker and to elucidate the chemical structure in complex matrices such as TCM formulations [3]. In this study, liquid chromatography coupled to quadruple time of flight (q-TOF) mass spectrometry was used for identification of chemical constituents, selection and quantification of analytical markers for quality control of YANG XIN® formulation.

1.3 Research objectives

1. Development of metabolite fingerprinting and profiling method of Traditional Chinese Medicine based formulations from two vendors to assess their quality using LC-MS and NMR.
2. Validation of the LC-MS quantitative method for analytical markers of YANG XIN® formulation from two vendors.

Chapter 2: Metabolite fingerprinting and profiling using LC-MS and NMR

2.1 Introduction

Chromatographic fingerprinting and chemical profiling of TCM products have proved to be a favorable approach to ensure quality control of herbal preparations and to distinguish the herbal preparations from other closely related species [9,10] by providing an overall picture of all the components of TCM formulations [5].

So far, chromatographic fingerprints have been globally accepted as efficient tools for quality control of TCM products [11] and authentication and identification of herbal products [12]. Therefore, many agencies such as the World Health Organization (WHO), the Food and Drug Administration (FDA), and the European Medicines Agency (EMA) recommend the use of chromatographic fingerprinting to verify herbal stability and consistency [13]. In 1991, the WHO introduced and accepted the chromatographic fingerprinting for herbal medicine quality assessment [14]. Through the past years, many methods have been established for chromatographic fingerprinting including thin layer chromatography (TLC), capillary electrophoresis (CE), gas chromatography (GC), high performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) [14,15]. Among them, the LC method is considered as the best option [13], because in the United States and Europe the herbal medicine monograph reports are primarily based on HPLC fingerprints [9]. Thus, liquid chromatography – mass spectrometry (LC-MS) has grown into the best technique for analyzing TCM products [15].

However, there are certain limitations with the use of the LC-MS technique for fingerprinting, like chromatographic selectivity which can be altered by, e.g. dwell volume,

gradient flow, temperature, and the age and the brand of the column. Another limitation is signal intensity which can be biased by instrument parameters or the detector's range of linearity. Therefore, such limitations can be overcome with the use of a different complementary technique like NMR fingerprints in which the ^{1}H NMR spectroscopy is used to generate a chemical fingerprint for herbal samples and their finished products. The main advantage of using NMR fingerprinting is its great reproducibility but its limitations are low sensitivity [16], expensive price, need for special environment, and training.

2.2 Materials and methods

2.2.1 Chemicals

LC-MS grade water and methanol, and HPLC grade dichloromethane were purchased from Thermo Fisher (Fair Lawn, NJ, USA). Formic acid was bought from Sigma-Aldrich (St. Louis, MO, USA). Seventeen standard compounds were purchased from the following suppliers: ophiopogonanone A (A Chemtek, Inc., Worcester, MA, USA), cistanoside D (AApin Chemicals Limited, Oxon, OX14 4RU, UK), sibiricoses A5 and forsythoside A (Analyticon Discovery, Potsdam, Germany), neoxanthine (BOC Sciences, Shirley, NY, USA), poricoic acid A, batatasin III, and pinusolide (ChemFaces, Wuhan, Hubei, PRC), α-asarone, formononetin, ginsenoside Rf, and swertisin (Chromadex, Irvine, CA, USA), armepavine (Ryan Scientific Inc, Mount Pleasant, SC, USA), racemic mixture of 3-butylidenephthalide, schizandrin, and β-amyrin (Chromadex, Irvine, CA, USA), and 4,5-dicaffeoylquinic acid (AvaChem Scientific, San Antonio, TX, U.S.A). Three samples of YANG XIN® capsules (Nourish the fire; Stock No. 1017-1), concentrated extract 2014-005_1A, and concentrated extract 2014-005_1B were supplied by Nature's Sunshine Products, Inc. and analyzed in this study.

2.2.2 Extraction method

Eighteen plants supplied by Nature's Sunshine Products, Inc., in addition to YANG XIN® capsules, concentrated extracts 2014-005_1A, and 2014-005_1B were extracted according to the following extraction method:

2.2.2.1. Maceration extraction with dichloromethane and methanol

Thirty grams (30 g) of each plant material, concentrated extracts 2014-005_1A and 2014-005_1B, and YANG XIN® capsules were extracted with 300 mL of dichloromethane. The mixture was stirred using a mechanical shaker for 24 hours. The macerate was allowed to settle for 30 minutes. The extract was then filtered through two filter papers Whatman No. 4 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and concentrated under vacuum in a rotary evaporator (Büchi, Flawil, Switzerland) at 40 °C. The concentrated extract was transferred into a small vial and placed under a nitrogen evaporator (Organomation Associate Inc., Berlin, MA, USA) for complete dryness. After filtration, the macerate was further extracted with methanol, filtered and dried using the same procedure described above.

2.2.3 LC-ESI-MS conditions for chemical fingerprinting and profiling

Ten (10) µl of the plant extract or two (2) µl of reference standard solutions were separated on reversed phase column Poroshell 120 EC-C18, 2.7 µm x 2.1 mm x 150 mm (Agilent Technologies, Inc, Little Falls, DE, USA). Two solvents (A and B) were used in the analysis. Solvent (A) was composed of water with 0.1% formic acid and solvent (B) was composed of methanol with gradient elution as follows: 0-6 min, 0 to 10% B; 6-8 min, 10 to 60% B; 8-40 min, 60 to 100% B; 40-42, 100% to 0 % and followed by 3 minutes post run with 0% solvent B. The total run time of the analysis was 45 minutes at flow rate of 0.2 ml/min and column temperature of 25 °C. A rapid resolution LC 1200 series coupled to 6520 accurate mass Q-TOF spectrometer

(Agilent Technologies, Little Falls, DE, USA) equipped with dual electrospray ionization (ESI) source was used for the analysis. Data acquisitions were collected under both positive (+) and negative (-) mode of ionization using full spectrum scan analysis for qualitative and quantitative analyses of the samples. For the mass spectrometer conditions, the nebulizing and drying gases were nitrogen supplied at a flow rate of 25 Psig and 10 ml/min, respectively. Drying gas temperature was 350 °C. Ionization source was dual ESI (electrospray ionization) with a capillary voltage of 3200 V. Fragmentor voltage was 175 V. Identification of compounds was performed using Agilent Mass Hunter Qualitative Analysis software, version B.06.00.

For the instrument's parameters when equipped with atmospheric pressure chemical ionization (APCI) ion source, the method of detection of β-amyrin consisted of isocratic 100% methanol. Total run time of the analysis was 20 minutes at a flow rate of 0.2 ml/min and column temperature of 25 °C. A rapid resolution LC 1200 series coupled to a 6520 accurate mass Q-TOF spectrometer (Agilent Technologies, Little Falls, DE, USA) equipped with APCI source was used for the analysis. Data acquisitions were collected under positive mode of ionization using full spectrum scan analysis for qualitative and quantitative analyses of the samples. For the mass spectrometer conditions, the nebulizing and drying gases were nitrogen supplied at a flow rate of 60 Psig and 10 ml/min, respectively. The drying gas temperature was 350 °C. Vaporizer temperature 450 °C with capillary voltage of 3500 V. The fragmentor voltage was 175 V. Identification of compounds was performed using Agilent Mass Hunter Qualitative Analysis software, version B.06.00.

2.2.4 ^{1}H NMR spectroscopy conditions for chemical fingerprinting

One hundred milligrams of the dried extract was dissolved in 1 mL of DMSO-d_6 (Sigma Aldrich, St. Louis, MO, USA) and filtered through glass wool fiber. NMR analysis was performed

using a Varian 400 MHz NMR spectrometer (Stevens Creek Boulevard, Santa Clara, CA) operating at a proton NMR experiment with frequency of 400 MHz. Scan rate was 512 with total time of 30 minutes. The residual solvent DMSO-d_6 was used as a referenced signal at 2.2 ppm.

2.2.5 Sample preparation for LC-MS analysis

The plant extract was redissolved in methanol or dimethyl sulfoxide (DMSO) depending on the solubility of the extract in a concentration of 5 mg/ml, then filtered through a syringe connected to a syringe filter (Whatman 0.45 μm PTFE filter w/GMF, NJ) and diluted 20 times with methanol to give a concentration 0.25 mg/mL then injected into the LC-MS system. Samples were stored in -20 °C freezer.

2.3 Results and discussion

2.3.1 Identification of chemical markers in YANG XIN® formulations using LC-MS based fingerprinting chromatograms

2.3.1.1. (+) ESI-LC-MS analysis

Peaks corresponding to chemical markers in Figures 2-37 were identified using the LC-MS and are listed in Table 1.

2.3.1.2. (-) ESI-LC-MS analysis

Peaks corresponding to chemical markers in Figures 2-37 were identified using the LC-MS and are listed in Table 1.

2.3.2 Strategy used for selection of chemical markers in plant ingredients

A literature search on the compounds isolated from each plant ingredient was carried out in Scifinder and PubMed. After analysis of the literature, known compounds were dereplicated in each plant material listed in Table 1 and Figure 1. The identification of compounds relied also on

the data of accurate mass measurement, molecular formula and double bond equivalent (DBE), score, and error (ppm) of the measurement as well as the comparison of the data with the previously published data for the compounds [9] (Figure 38). A potential marker per plant was compared with each one of the markers present in every plant ingredient of the formulations to avoid duplicity of the markers. Another relevant characteristic of the marker is its commercial availability. This allowed for the determination of the presence of each plant ingredient in the formulations. Table 3 lists the markers selected per plant ingredient and their commercial availability.

A comparison between the accurate mass of the proposed chemical marker with the corresponding accurate mass of chemical constituents of plant raw material is included in the formula and the three samples of the formulation. Further confirmation of the selected chemical marker was done by matching of retention time, molecular formula, and double bond equivalent (DBE) of each chemical marker in the plant materials and extracts 2014-005_1A and 2014-005_1B and YANG XIN® capsules, and the reference standard (Figure 39). Figures 2-37 provide the total ion chromatogram of the plants and the extracted ion chromatogram of chemical markers selected per each plant.

Eleven markers out of 18 markers have been selected for the first time as chemical markers to represent the plants in the YANG XIN® formulations. Two markers, forsythoside A and β-amyrin, were present in more than one plant present in YANG XIN® formulations. Forsythoside A was found in *Rehmannia glutinosa* and *Cistanche salsa* whereas β-amyrin was present in *Lycium barbarum*, *Panax ginseng*, and *Amber*. Because other specific compounds to these 5 plant ingredients were not commercially available, forsythoside A was selected as an analytical marker for *Rehmannia glutinosa* and β-amyrin for *Amber*. The identification of β-amyrin was carried on APCI because the compound was non-polar and did not ionize in the ESI mode. The chemical

marker polygonatine A was not commercially available due to the failure in its chemical synthesis by two vendors.

Table 1 LC-MS identification of the analytical markers

Plant	Family	Part	t_R	Accurate mass	Exact mass	Formula	DBE	Error (ppm)	Score	Marker
Angelica sinensis (Tang-kuei root)	Apiaceae	Root	19.91	188.0838	188.0837	$C_{12}H_{12}O_2$	7	-0.47	99.94	Z-Butylidenephthalide
Cuscuta chinensis (Cuscuta seed)	Convolvulaceae	Seed	11.22	516.1273	516.1268	$C_{25}H_{24}O_{12}$	14	-1.06	97.61	4,5 Dicaffeoylquinic acid
Nelumbo nucifera (Lotus seed)	Nelumbonaceae	Seed	10.00	313.1679	313.1678	$C_{19}H_{23}NO_3$	9	-0.42	99.04	Armepavine
Dioscorea batatas (Dioscorea rhizome)	Dioscoreaceae	Rhizome	14.50	244.1099	244.1099	$C_{15}H_{16}O_3$	8	0.29	99.81	Batatasin III
Cistanche salsa (Cistanche stem)	Orobanchaceae	Stem	11.58	652.2372	652.2367	$C_{31}H_{40}O_{15}$	12	-0.78	99.14	Cistanoside D
Astragalus membranaceus (Astragalus root)	Fabaceae	Root	16.98	268.0734	268.0736	$C_{16}H_{12}O_4$	11	0.62	98.6	Formononetin
Rehmannia glutinosa (Rehmannia root tuber)	Orobanchaceae	Root Tuber	10.69	624.2043	624.2054	$C_{29}H_{36}O_{15}$	12	1.78	97.44	Forsythoside A
Panax ginseng (Ginseng root)	Araliaceae	Root	18.53	800.4961	800.4922	$C_{42}H_{72}O_{14}$	7	0.81	96.06	Ginsenoside Rf
Lycium barbarum (Lycium fruit)	Solanaceae	Fruit	36.80	600.4178	600.4179	$C_{40}H_{56}O_4$	13	0.04	99.8	Neoxanthin
Ophiopogon japonicas (Ophiopogon root tuber)	Asparagaceae	Root Tuber	23.78	328.0941	328.0947	$C_{18}H_{16}O_6$	11	1.72	95.9	Ophiopogonanone A
Biota orientalis (Biota seed)	Cupressaceae	Seed	23.70	346.215	346.2144	$C_{21}H_{30}O_4$	7	-1.83	92.97	Pinusolide
Polygonatum sibiricum (Polygonatum rhizome)	Asparagaceae	Rhizome	9.60	165.0788	165.0790	$C_9H_{11}NO_2$	5	1.1	87.35	Polygonatine A
Poria cocos (Hoelen sclerotium)	Polyporaceae	Sclerotium	30.99	498.3349	498.3345	$C_{31}H_{46}O_5$	9	-0.84	98.96	Poricoic Acid A
Schisandra chinensis (Schizandra fruit)	Schisandraceae	Fruit	17.30	432.2147	432.2148	$C_{24}H_{32}O_7$	9	0.18	99.65	Schisandrol A
Polygala tenuifolia (Polygala root)	Polygalaceae	Root	10.33	518.1638	518.1636	$C_{22}H_{30}O_{14}$	8	-0.5	99.54	Sibiricoses A5
Zizyphus spinosa (Jujuba seed)	Rhamnaceae	Seed	11.45	446.1217	446.1213	$C_{22}H_{22}O_{10}$	12	-.0.94	98.95	Swertisin
Amber (Succinum amber)	Unclassified	Unclassified	10.71	426.3861	426.3862	$C_{30}H_{50}O$	6	0.05	99.76	β-Amyrin
Acorus gramineus (Acorus rhizome)	Acoraceae	Rhizome	18.32	208.1098	208.1099	$C_{12}H_{16}O_3$	5	0.87	99.64	α-Asarone

t_R: Retention time; DBE: Double bond equivalent

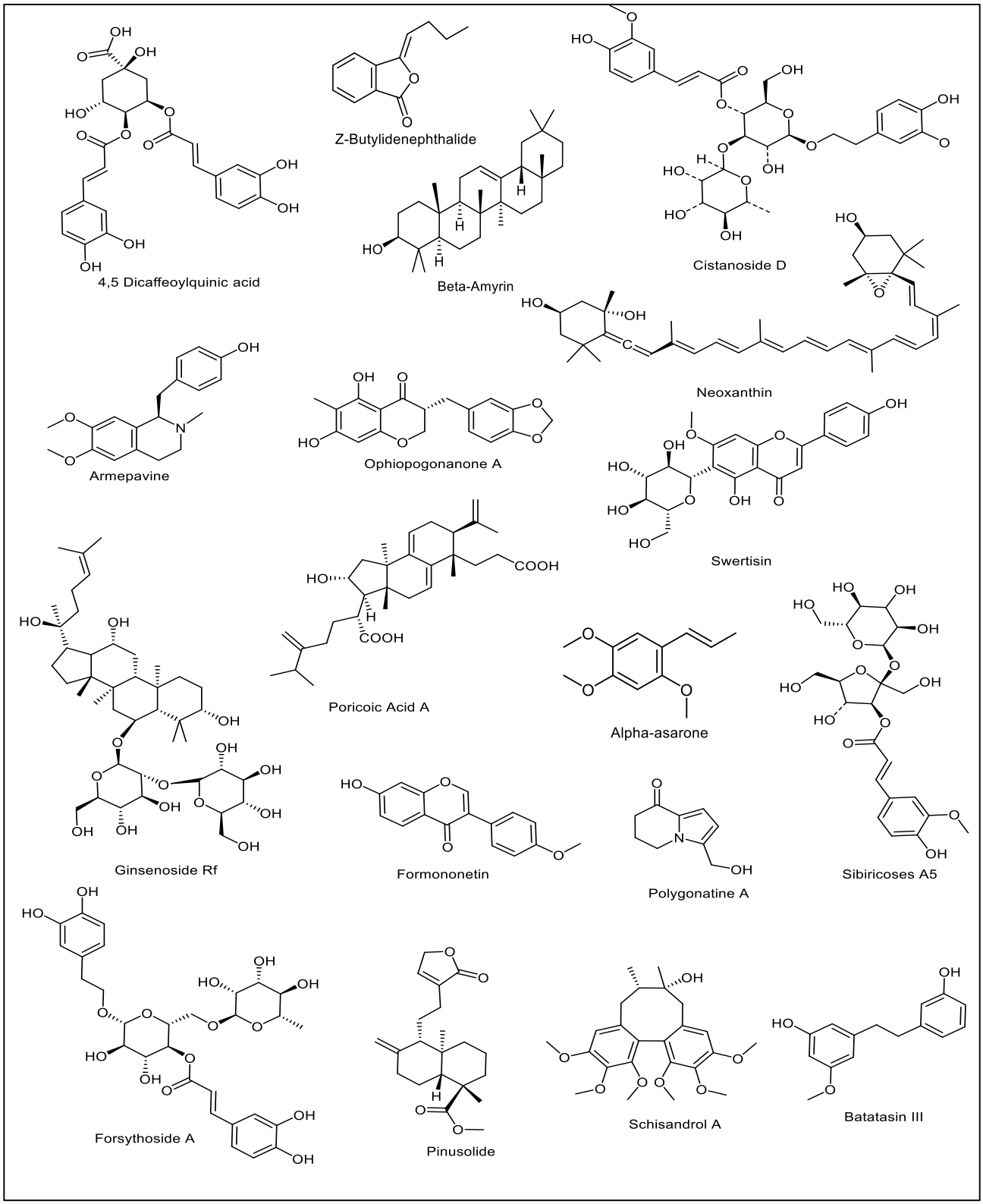

Figure 1. Chemical structure of the analytical markers

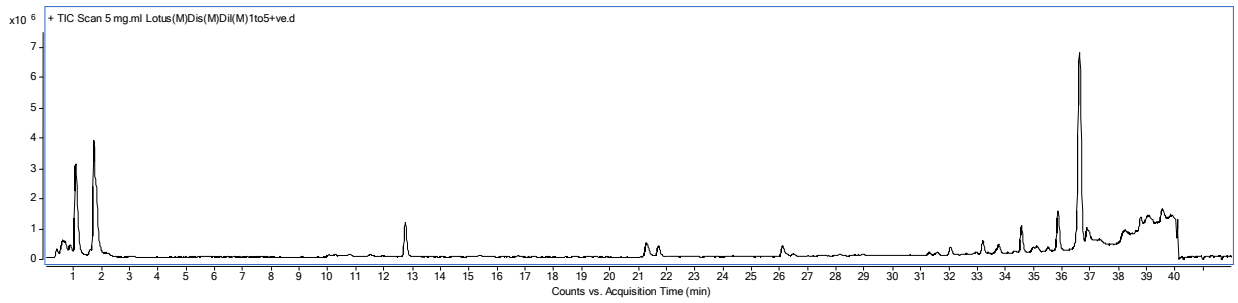

Figure 2. Total ion chromatogram of *Nelumbo nucifera* (Lotus seed)

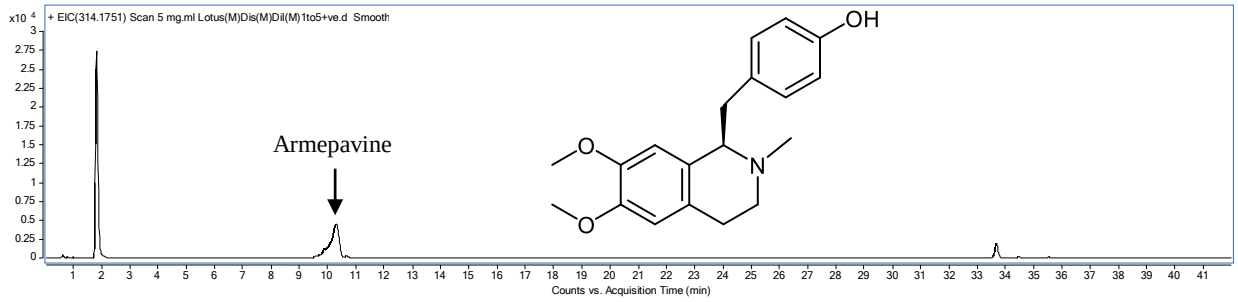

Figure 3. Extracted ion chromatogram of armepavine from *Nelumbo nucifera* (Lotus seed). Methanol extract; ESI; positive ionization mode.

Figure 2 shows the total ion chromatogram obtained from the methanol extract of *Nelumbo nucifera* seeds in the positive ionization mode. Figure 3 is the extracted ion chromatogram of the sample from Figure 2 monitoring for the molecular ion $[M+H]^+$ at 314.1751 *m/z*. The results indicate three different peaks of equivalent ion (314.1751 *m/z*) which indicate three possible isomers of armepavine. A chromatographic comparison with the reference standard shows that the peak at retention time 10 minutes was armepavine. The excellent ionization of armepavine in the positive mode is due to the presence of a basic nitrogen atom.

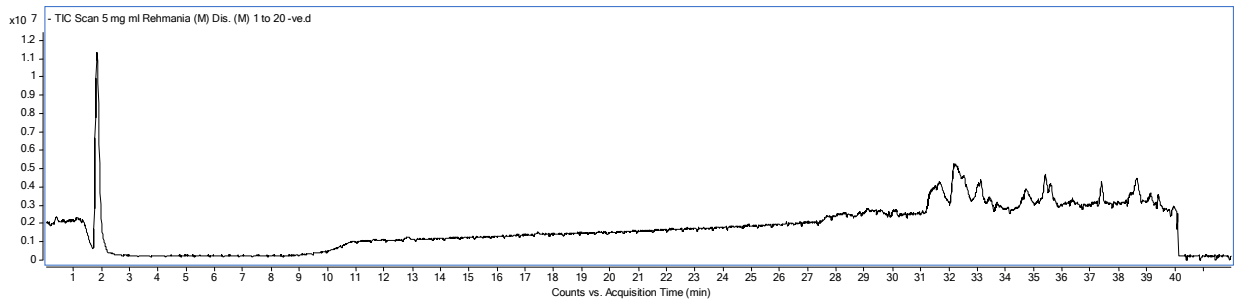

Figure 4. Total ion chromatogram of *Rehmannia glutinosa* (Rehmannia root tuber)

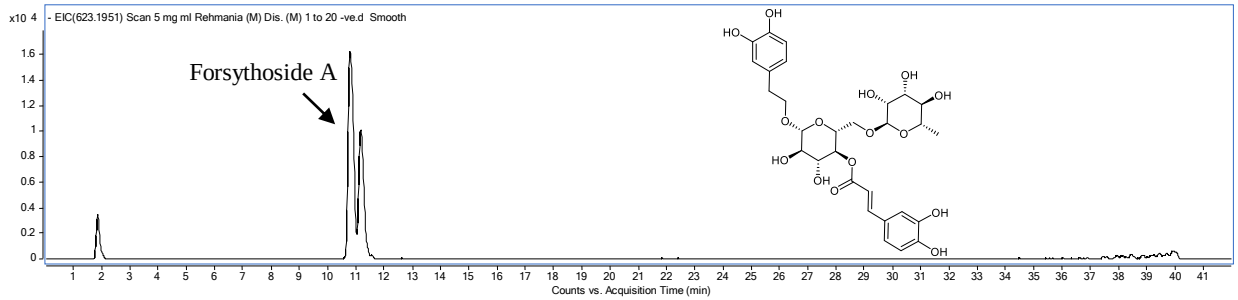

Figure 5. Extracted ion chromatogram of forsythoside A from *Rehmannia glutinosa* (Rehmannia root tuber). Methanol extract; ESI; negative ionization mode.

Figure 4 shows the total ion chromatogram obtained from the methanol extract of *Rehmannia glutinosa* root in the negative ionization mode. Figure 5 is the extracted ion chromatogram of the sample from Figure 4 monitoring for the molecular ion $[M-H]^-$ at 623.1951 *m/z*. The results indicate three different peaks of equivalent ion (623.1951 *m/z*) which indicate three possible isomers of Forsythoside A. A chromatographic comparison with the reference standard shows that the peak at retention time 10.9 minutes was Forsythoside A. The excellent ionization of Forsythoside A in the negative mode is due to the presence of acidic catechol groups.

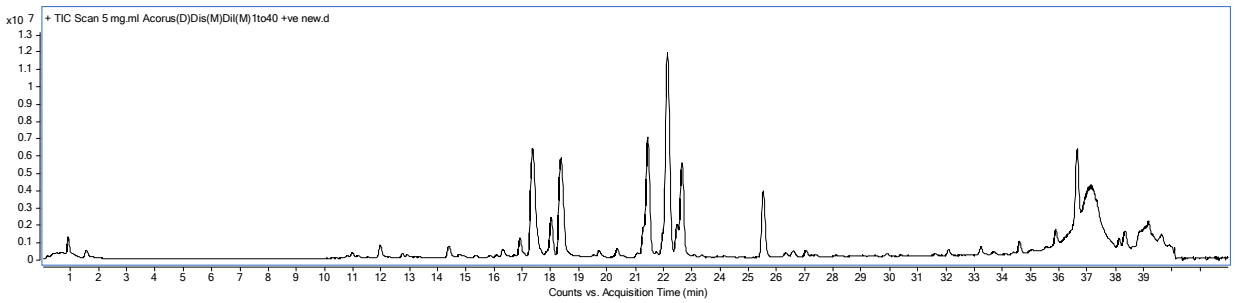

Figure 6. Total ion chromatogram of *Acorus gramineus* (Acorus rhizome)

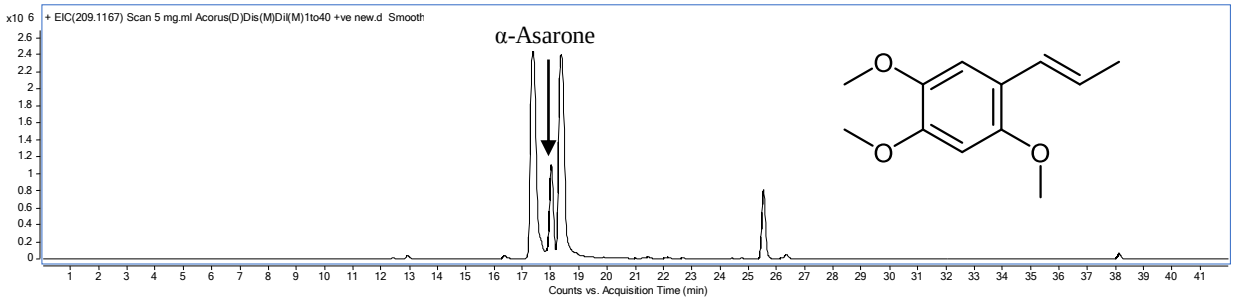

Figure 7. Extracted ion chromatogram of α-asarone from *Acorus gramineus* (Acorus rhizome). Dichloromethane extract; ESI; positive ionization mode.

Figure 6 shows the total ion chromatogram obtained from the dichloromethane extract of *Acorus gramineus* rhizome in the positive ionization mode. Figure 7 is the extracted ion chromatogram of the sample from Figure 6 monitoring for the molecular ion $[M+H]^+$ at 209.1167 *m/z*. The results indicate four different peaks of equivalent ion (209.1167 *m/z*) which indicate four possible isomers of α-asarone. A chromatographic comparison with the reference standard shows that the peak at retention time 18 minutes was α-asarone. The excellent ionization of α-asarone in the positive mode is probably due to the presence of alkene group.

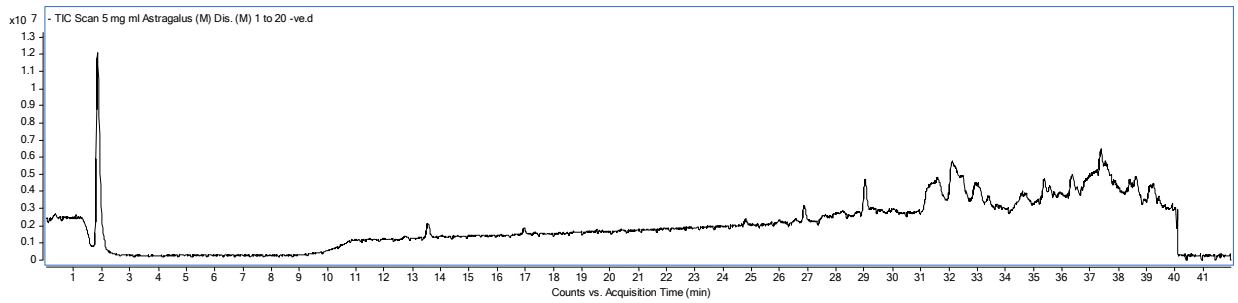

Figure 8. Total ion chromatogram of *Astragalus membranaceus* (Astragalus root)

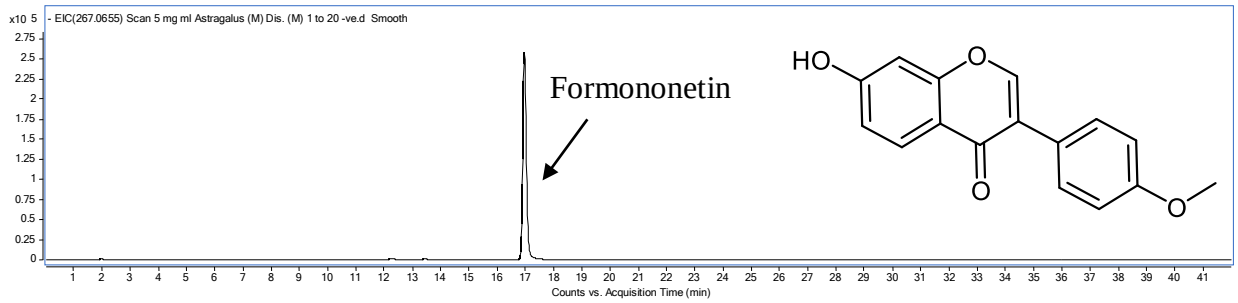

Figure 9. Extracted ion chromatogram of formononetin from *Astragalus membranaceus* (Astragalus root). Methanol extract; ESI; negative ionization mode.

Figure 8 shows the total ion chromatogram obtained from the methanol extract of *Astragalus membranaceus* root in the negative ionization mode. Figure 9 is the extracted ion chromatogram of the sample from Figure 8 monitoring for the molecular ion $[M-H]^-$ at 267.0655 *m/z*. The results indicate one peaks of equivalent ion (267.0655 *m/z*) which probably indicate formononetin. A chromatographic comparison with the reference standard shows that the peak at retention time 17 minutes was formononetin. The excellent ionization of formononetin in the negative mode is due to the presence of acidic hydroxyl group.

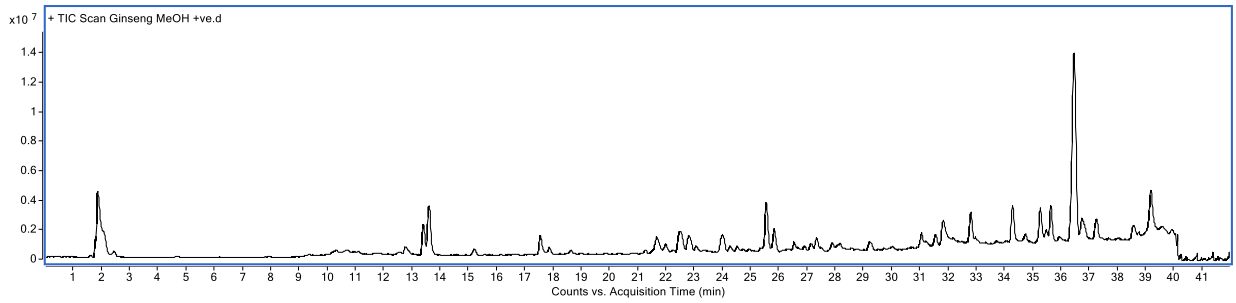

Figure 10. Total ion chromatogram of *Panax ginseng* (Ginseng root)

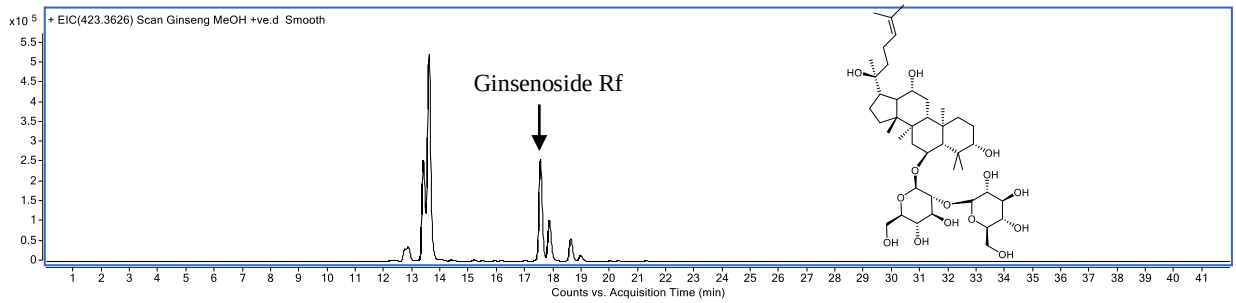

Figure 11. Extracted ion chromatogram of ginsenoside Rf from *Panax ginseng* (Ginseng root). Methanol extract; ESI; positive ionization mode.

Figure 10 shows the total ion chromatogram obtained from the methanol extract of *Panax ginseng* root in the positive ionization mode. Figure 11 is the extracted ion chromatogram of the sample from Figure 10 monitoring for the molecular ion $[(M+H)\text{-}GluGlu\text{-}H_2O]^+$ at 423.3626 *m/z*. The results indicate multiple different peaks of equivalent ion (423.3626 *m/z*) which indicate multiple possible isomers of ginsenoside Rf. A chromatographic comparison with the reference standard shows that the peak at retention time 17.6 minutes was ginsenoside Rf. The excellent ionization of ginsenoside Rf in the positive mode is probably due to the presence of alkene group.

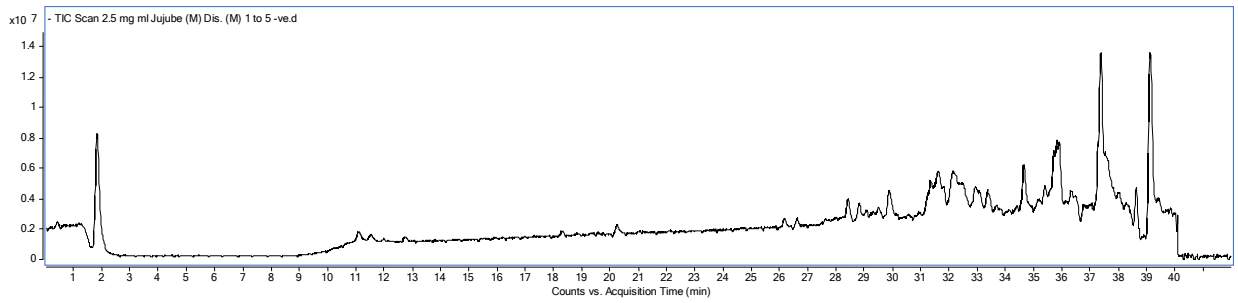

Figure 12. Total ion chromatogram of *Zizyphus spinosa* (Jujuba seed)

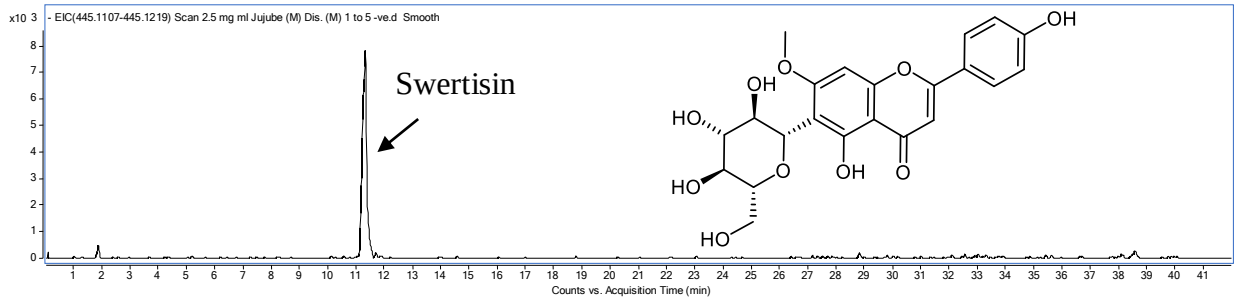

Figure 13. Extracted ion chromatogram of swertisin from *Zizyphus spinosa* (Jujuba seed). Methanol extract; ESI; negative ionization mode.

Figure 12 shows the total ion chromatogram obtained from the methanol extract of *Zizyphus spinosa* seed in the negative ionization mode. Figure 13 is the extracted ion chromatogram of the sample from Figure 12 monitoring for the molecular ion $[M-H]^-$ at 445.1163 *m/z*. The results indicate one peaks of equivalent ion (445.1163 *m/z*) which probably indicates swertisin. A chromatographic comparison with the reference standard shows that the peak at retention time 11.5 minutes was swertisin. The excellent ionization of swertisin in the negative mode is due to the presence of acidic hydroxyl groups.

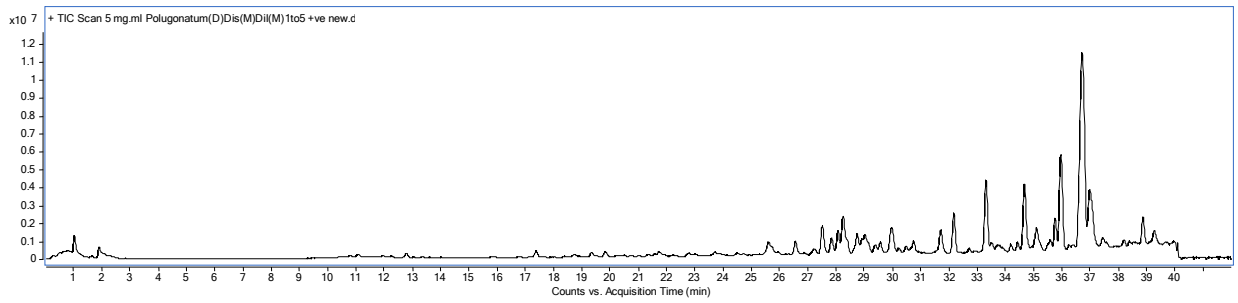

Figure 14. Total ion chromatogram of of *Polygonatum sibiricum* (Polygonatum rhizome)

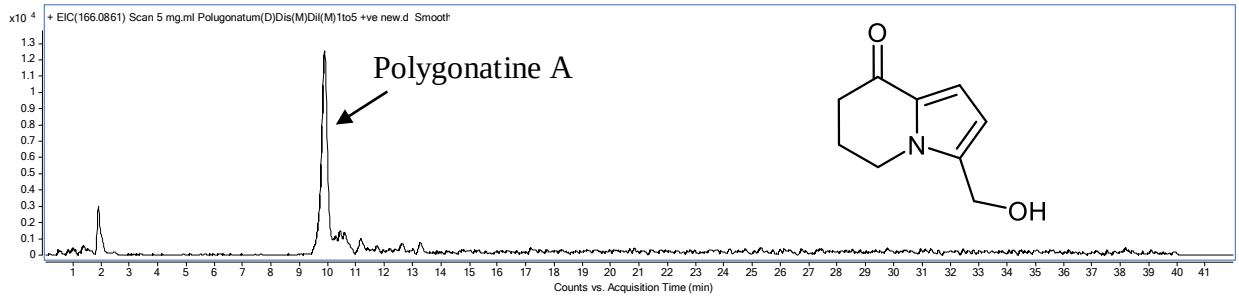

Figure 15. Extracted ion chromatogram of polygonatine A from *Polygonatum sibiricum* (Polygonatum rhizome). Dichloromethane extract; ESI; positive ionization mode.

Figure 14 shows the total ion chromatogram obtained from the dichloromethane extract of *Polygonatum sibiricum* rhizome in the positive ionization mode. Figure 15 is the extracted ion chromatogram of the sample from Figure 14 monitoring for the molecular ion $[M+H]^+$ at 166.0861 *m/z*. The results indicate multiple different peaks of equivalent ion (166.0861 *m/z*) which indicate multiple possible isomers of polygonatine A. A chromatographic comparison with the reference standard shows that the peak at retention time 10 minutes was polygonatine A. The excellent ionization of polygonatine A in the positive mode is due to the presence of nitrogen atom.

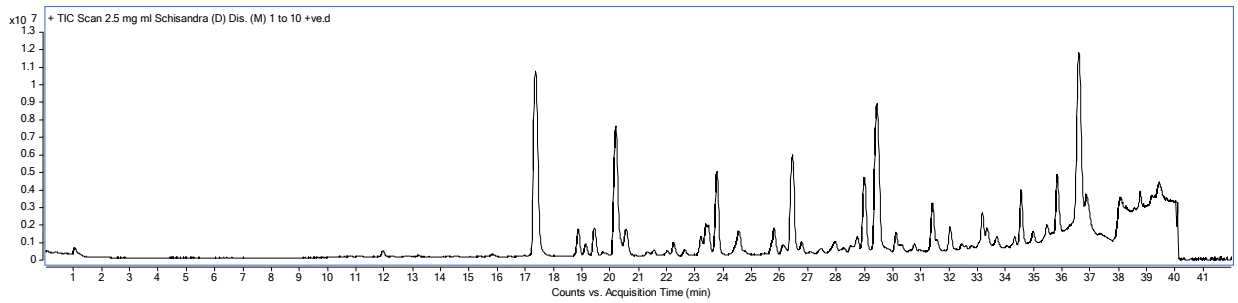

Figure 16. Total ion chromatogram of *Schisandra chinensis* (Schizandra fruit)

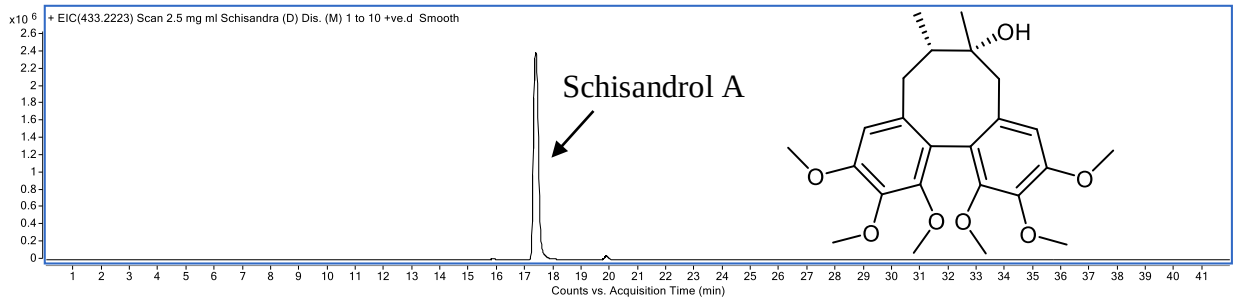

Figure 17. Extracted ion chromatogram of schisandrol A from *Schisandra chinensis* (Schizandra fruit). Dichloromethane extract; ESI; positive ionization mode.

Figure 16 shows the total ion chromatogram obtained from the dichloromethane extract of *Schisandra chinensis* fruit in the positive ionization mode. Figure 17 is the extracted ion chromatogram of the sample from Figure 16 monitoring for the molecular ion $[M+H]^+$ at 433.2223 *m/z*. The results indicate two different peaks of equivalent ion (433.2223 *m/z*) which indicate two possible isomers of schisandrol A. A chromatographic comparison with the reference standard shows that the peak at retention time 17.5 minutes was schisandrol A.

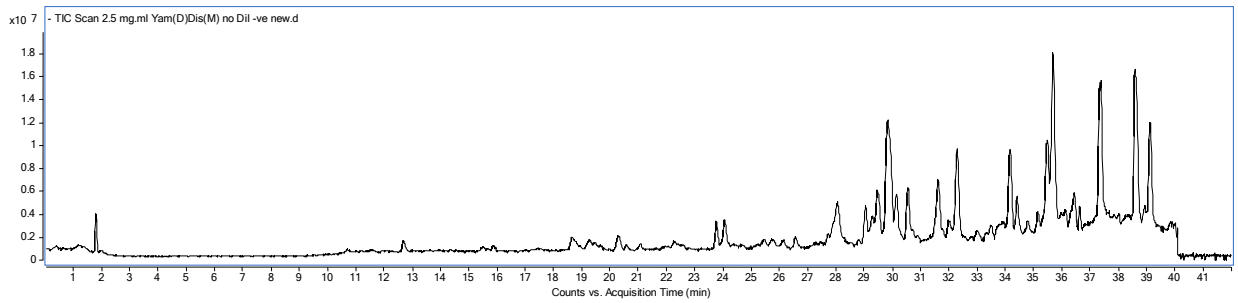

Figure 18. Total ion chromatogram of *Dioscorea batatas* (Dioscorea rhizome)

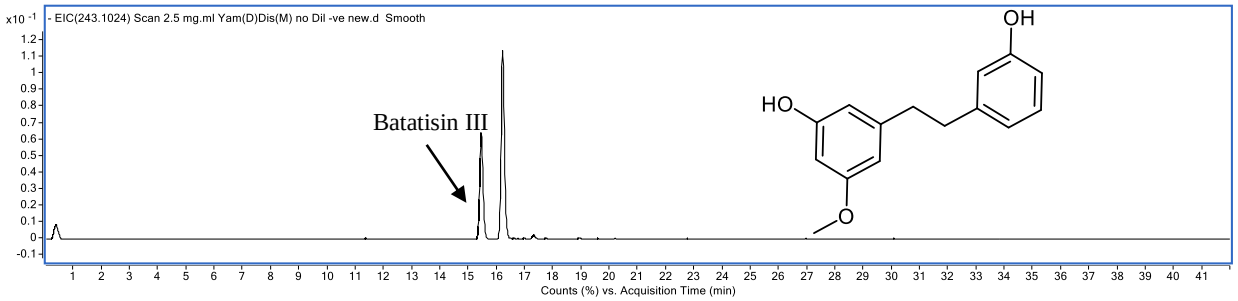

Figure 19. Extracted ion chromatogram of batatisin III from *Dioscorea batatas* (Dioscorea rhizome). Dichloromethane extract; ESI; negative ionization mode.

Figure 18 shows the total ion chromatogram obtained from the dichloromethane extract of *Dioscorea batatas* rhizome in the negative ionization mode. Figure 19 is the extracted ion chromatogram of the sample from Figure 18 monitoring for the molecular ion $[M-H]^-$ at 243.1024 *m/z*. The results indicate two different peaks of equivalent ion (243.1024 *m/z*) which indicate two possible isomers of batatisin III. A chromatographic comparison with the reference standard shows that the peak at retention time 15.5 minutes was batatisin III. The excellent ionization of batatisin III in the negative mode is due to the presence of acidic hydroxyl groups.

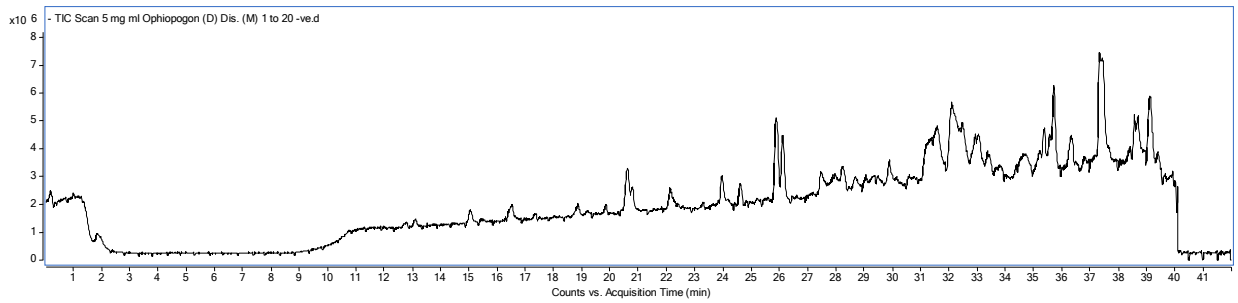

Figure 20. Total ion chromatogram of *Ophiopogon japonicas* (Ophiopogon root tuber)

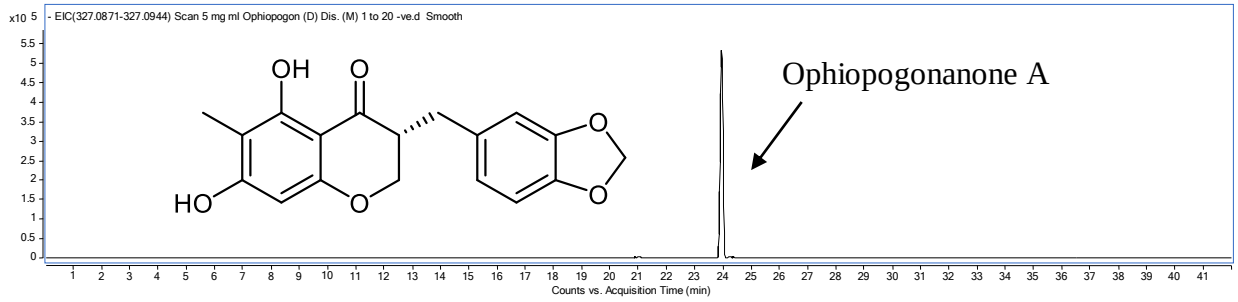

Figure 21. Extracted ion chromatogram of ophiopogonanone A from *Ophiopogon japonicas* (Ophiopogon root tuber). Dichloromethane extract; ESI; negative ionization mode.

Figure 20 shows the total ion chromatogram obtained from the dichloromethane extract of *Ophiopogon japonicas* root in the negative ionization mode. Figure 21 is the extracted ion chromatogram of the sample from Figure 20 monitoring for the molecular ion $[M-H]^-$ at 327.0908 *m/z*. The results indicate one peaks of equivalent ion (327.0908 *m/z*) which probably indicates ophiopogonanone A. A chromatographic comparison with the reference standard shows that the peak at retention time 24 minutes was ophiopogonanone A. The excellent ionization of ophiopogonanone A in the negative mode is due to the presence of acidic hydroxyl groups.

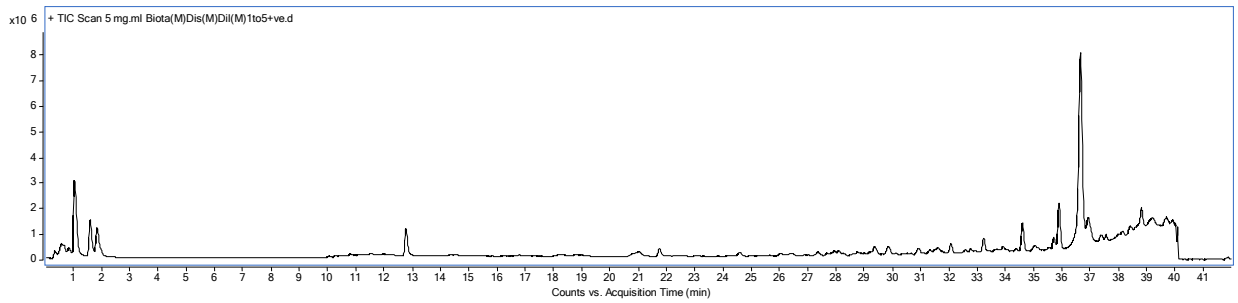

Figure 22. Total ion chromatogram of *Biota orientalis* (Biota seed)

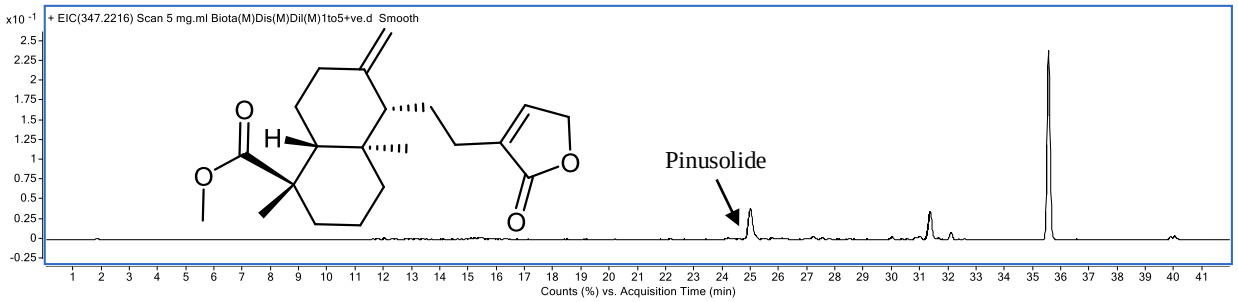

Figure 23 Extracted ion chromatogram of pinusolide from *Biota orientalis* (Biota seed). Methanol extract; ESI; positive ionization mode.

Figure 22 shows the total ion chromatogram obtained from the methanol extract of *Biota orientalis* seed in the positive ionization mode. Figure 23 is the extracted ion chromatogram of the sample from Figure 22 monitoring for the molecular ion $[M+H]^+$ at 347.2216 *m/z*. The results indicate three different peaks of equivalent ion (347.2216 *m/z*) which indicate three possible isomers of pinusolide. A chromatographic comparison with the reference standard shows that the peak at retention time 25 minutes was pinusolide. The excellent ionization of pinusolide in the positive mode is probably due to the presence of alkene group.

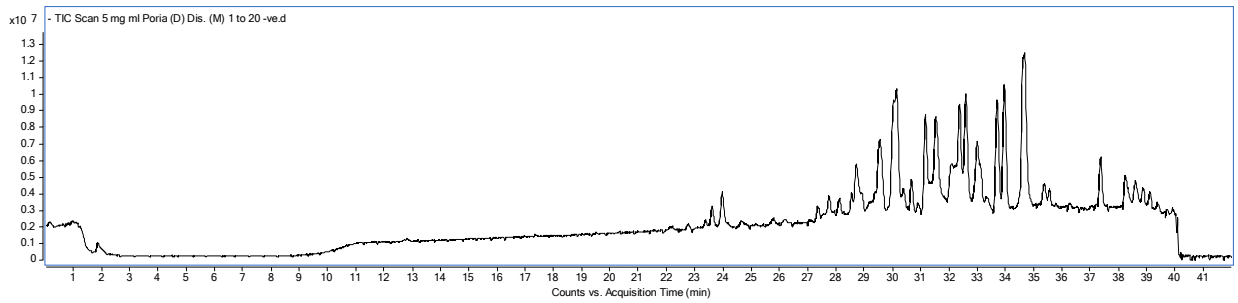

Figure 24. Total ion chromatogram of *Poria cocos* (Hoelen sclerotium)

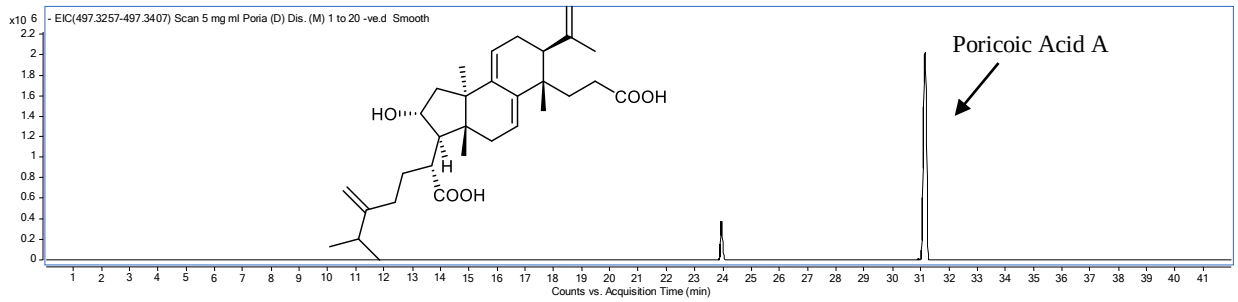

Figure 25. Extracted ion chromatogram of poricoic acid A from *Poria cocos* (Hoelen sclerotium). Dichloromethane extract; ESI; negative ionization mode.

Figure 24 shows the total ion chromatogram obtained from the dichloromethane extract of *Poria cocos* sclerotium in the negative ionization mode. Figure 25 is the extracted ion chromatogram of the sample from Figure 24 monitoring for the molecular ion $[M-H]^-$ at 497.3332 *m/z*. The results indicate two different peaks of equivalent ion (497.3332 *m/z*) which indicate two possible isomers of poricoic acid A. A chromatographic comparison with the reference standard shows that the peak at retention time 31 minutes was poricoic acid A. The excellent ionization of poricoic acid A in the negative mode is due to the presence of acidic carboxylic acid groups.

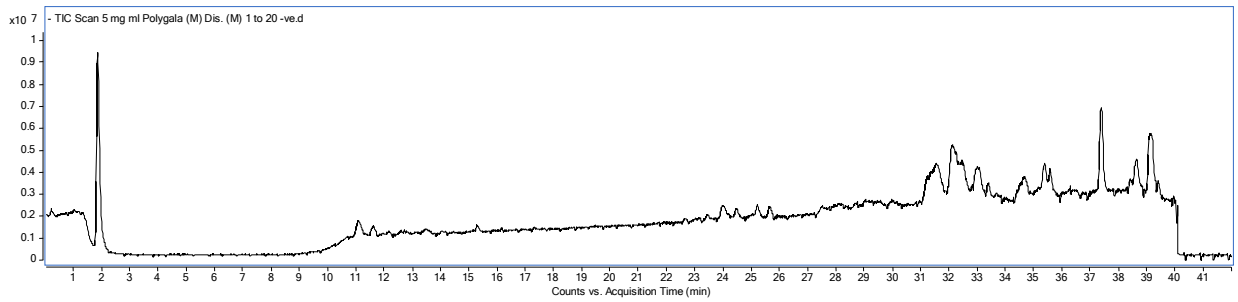

Figure 26. Total ion chromatogram of *Polygala tenuifolia* (Polygala root)

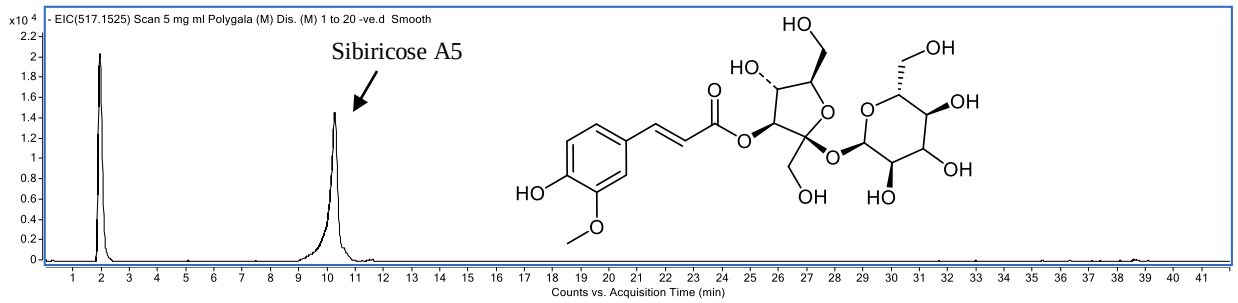

Figure 27. Extracted ion chromatogram of sibiricoses A5 from *Polygala tenuifolia* (Polygala root). Methanol extract; ESI; negative ionization mode.

Figure 26 shows the total ion chromatogram obtained from the methanol extract of *Polygala tenuifolia* root in the negative ionization mode. Figure 27 is the extracted ion chromatogram of the sample from Figure 26 monitoring for the molecular ion $[M-H]^-$ at 517.1525 *m/z*. The results indicate two different peaks of equivalent ion (517.1525 *m/z*) which indicate two possible isomers of sibiricoses A5. A chromatographic comparison with the reference standard shows that the peak at retention time 10.1 minutes was sibiricoses A5. The excellent ionization of sibiricoses A5in the negative mode is due to the presence of acidic hydroxyl group.

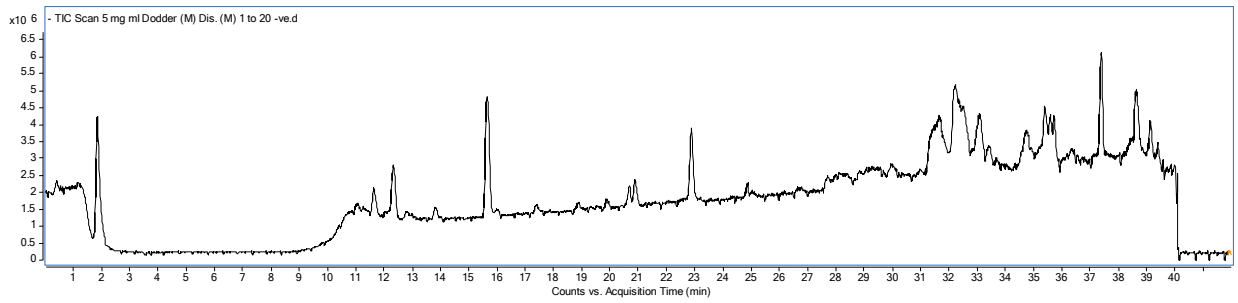

Figure 28. Total ion chromatogram of *Cuscuta chinensis* (Cuscuta seed)

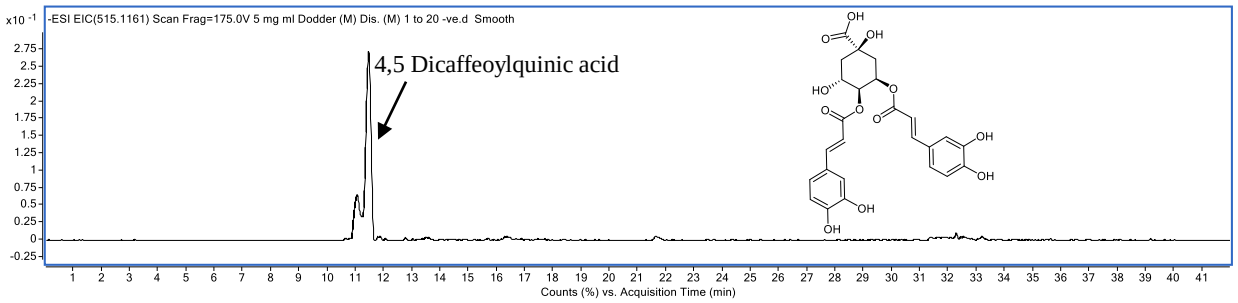

Figure 29. Extracted ion chromatogram of 4,5 dicaffeoylquinic acid from *Cuscuta chinensis* (Cuscuta seed). Methanol extract; ESI; negative ionization mode.

Figure 28 shows the total ion chromatogram obtained from the methanol extract of *Cuscuta chinensis* seed in the negative ionization mode. Figure 29 is the extracted ion chromatogram of the sample from Figure 28 monitoring for the molecular ion $[M-H]^-$ at 515.1161 *m/z*. The results indicate two different peaks of equivalent ion (515.1161 *m/z*) which indicate two possible isomers of 4,5 dicaffeoylquinic acid. A chromatographic comparison with the reference standard shows that the peak at retention time 11.6 minutes was 4,5 dicaffeoylquinic acid. The excellent ionization of 4,5 dicaffeoylquinic acid in the negative mode is due to the presence of acidic catechol groups.

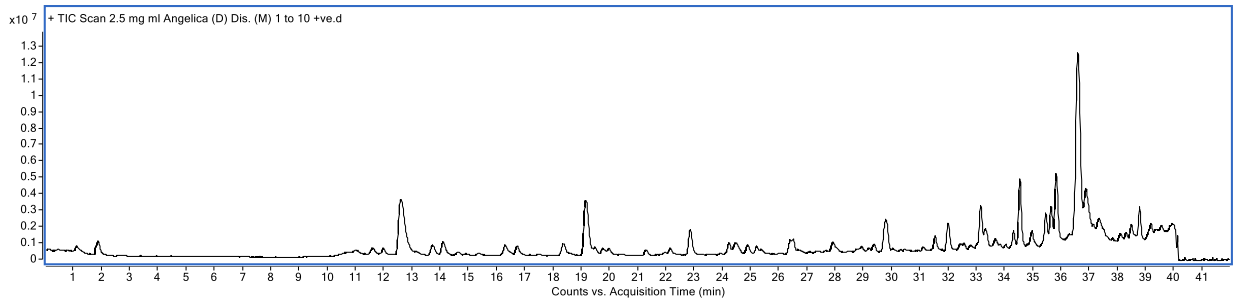

Figure 30. Total ion chromatogram of *Angelica sinensis* (Tangkuei root)

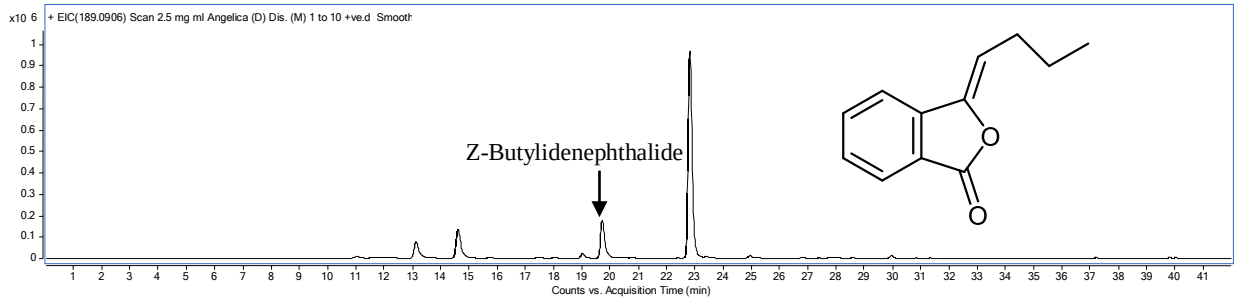

Figure 31. Extracted ion chromatogram of Z-butylidenephthalide from *Angelica sinensis* (Tangkuei root). Dichloromethane extract; ESI; positive ionization mode.

Figure 30 shows the total ion chromatogram obtained from the dichloromethane extract of *Angelica sinensis* root in the positive ionization mode. Figure 31 is the extracted ion chromatogram of the sample from Figure 30 monitoring for the molecular ion $[M+H]^+$ at 189.0906 *m/z*. The results indicate four different peaks of equivalent ion (189.0906 *m/z*) which indicate four possible isomers of Z-butylidenephthalide. A chromatographic comparison with the reference standard shows that the peak at retention time 10.9 minutes was Z-butylidenephthalide. The excellent ionization of Z-butylidenephthalide in the positive mode is probably due to the presence of alkene group.

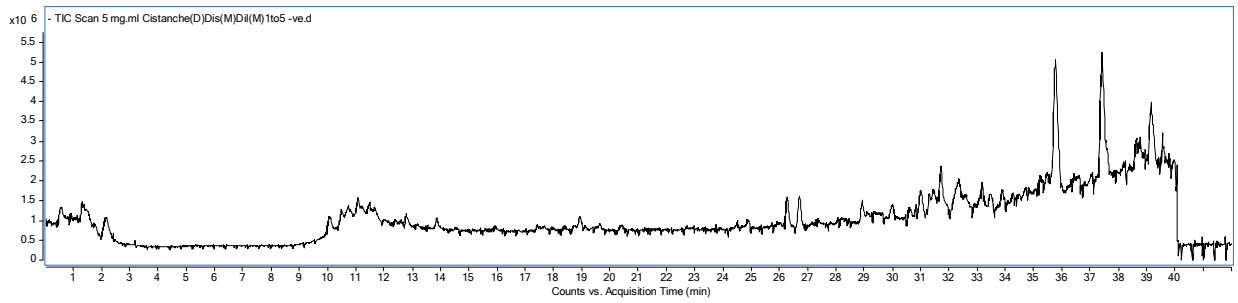

Figure 32. Total ion chromatogram of *Cistanche salsa* (Cistanche stem)

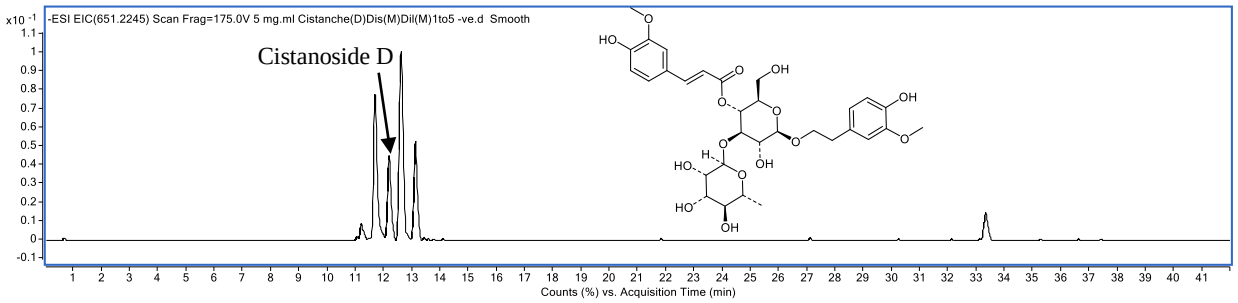

Figure 33. Extracted ion chromatogram of cistanoside D from *Cistanche salsa* (Cistanche stem). Dichloromethane extract; ESI; negative ionization mode.

Figure 32 shows the total ion chromatogram obtained from the dichloromethane extract of *Cistanche salsa* stem in the negative ionization mode. Figure 33 is the extracted ion chromatogram of the sample from Figure 32 monitoring for the molecular ion $[M-H]^-$ at 651.2245 *m/z*. The results indicate six different peaks of equivalent ion (651.2245 *m/z*) which indicate six possible isomers of cistanoside D. A chromatographic comparison with the reference standard shows that the peak at retention time 12.2 minutes was cistanoside D. The excellent ionization of cistanoside D in the negative mode is due to the presence of acidic hydroxyl groups.

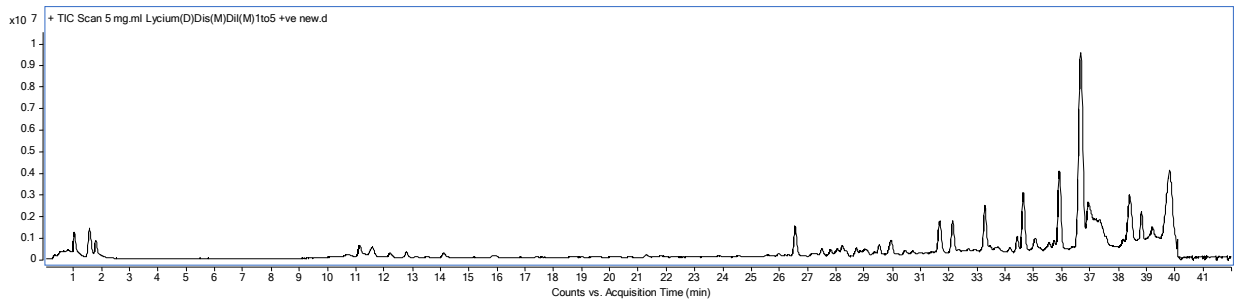

Figure 34. Total ion chromatogram of *Lycium barbarum* (Lycium fruit)

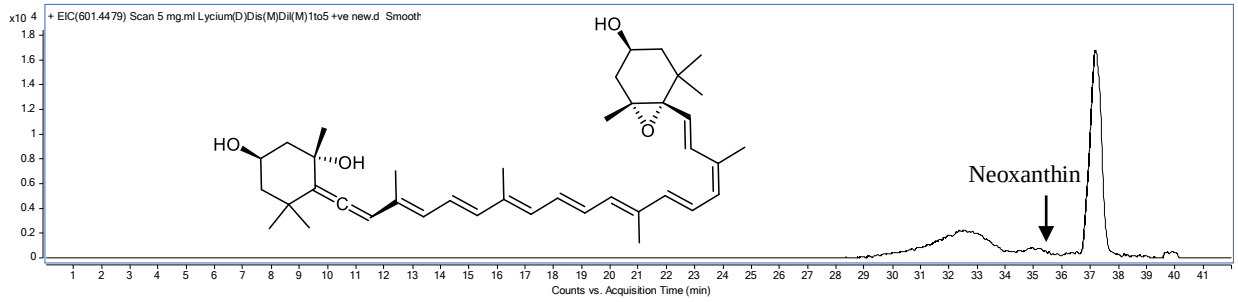

Figure 35. Extracted ion chromatogram of neoxanthin from *Lycium barbarum* (Lycium fruit). Dichloromethane extract; ESI; positive ionization mode.

Figure 34 shows the total ion chromatogram obtained from the dichloromethane extract of *Lycium barbarum* fruit in the positive ionization mode. Figure 35 is the extracted ion chromatogram of the sample from Figure 34 monitoring for the molecular ion $[M+H]^+$ at 601.4479 *m/z*. The results indicate four different peaks of equivalent ion (601.4479 *m/z*) which indicate four possible isomers of neoxanthin. A chromatographic comparison with the reference standard shows that the peak at retention time 35.7 minutes was neoxanthin. The excellent ionization of neoxanthin in the positive mode is probably due to the presence of alkene groups.

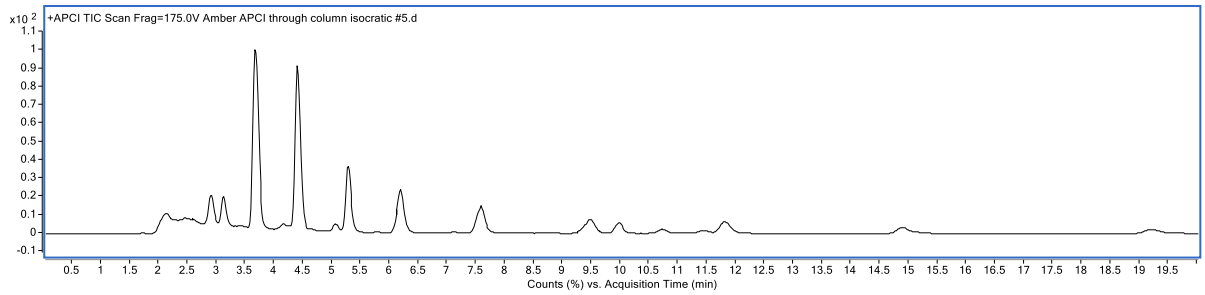

Figure 36. Total ion chromatogram of *Amber* (Succinum)

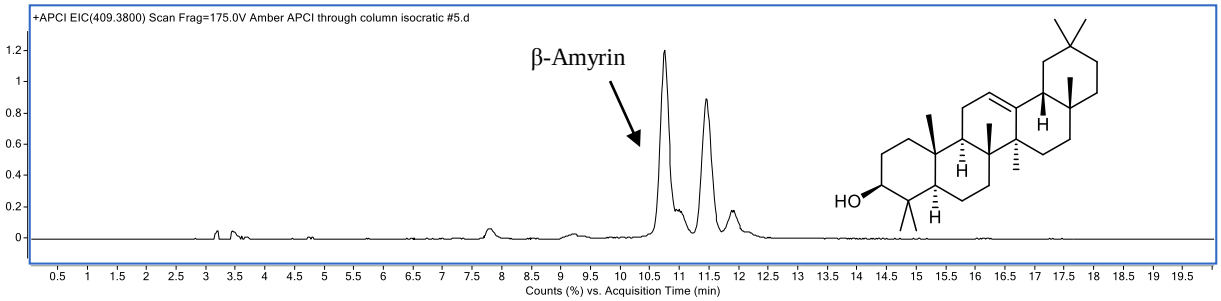

Figure 37. Extracted ion chromatogram of β-amyrin from *Amber* (Succinum). Dichloromethane extract; ESI; positive ionization mode.

Figure 36 shows the total ion chromatogram obtained from the dichloromethane extract of *Amber* in the positive ionization mode. Figure 37 is the extracted ion chromatogram of the sample from Figure 36 monitoring for the molecular ion $[(M+H)-H_2O]^+$ at 409.3800 *m/z*. The results indicate three different peaks of equivalent ion (409.3800 *m/z*) which indicate three possible isomers of β-amyrin. A chromatographic comparison with the reference standard shows that the peak at retention time 10.7 minutes was β-amyrin. The excellent ionization of β-amyrin in the positive mode is probably due to the presence of alkene group.

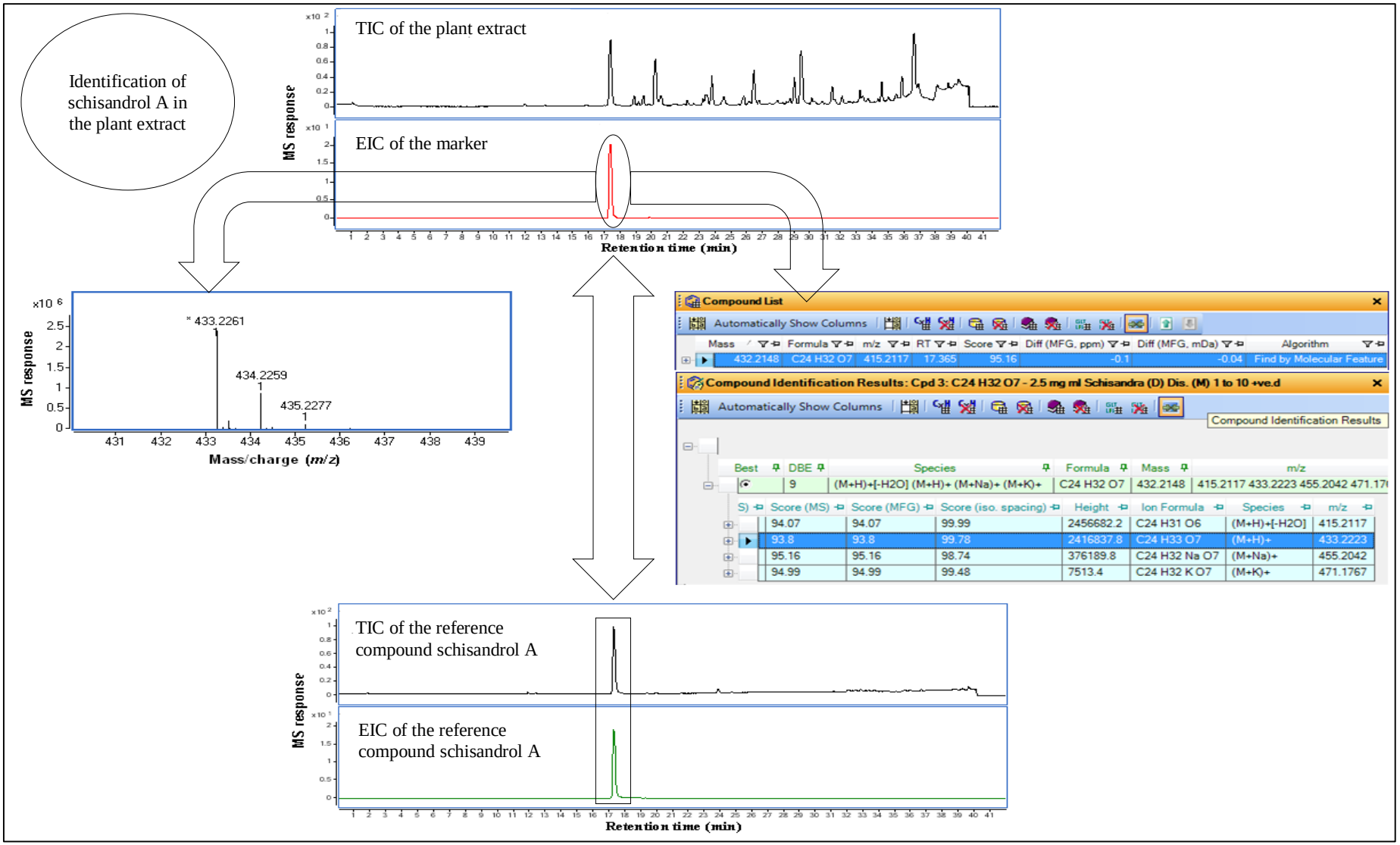

Figure 38. Identification of schisandrol A in the plant extract

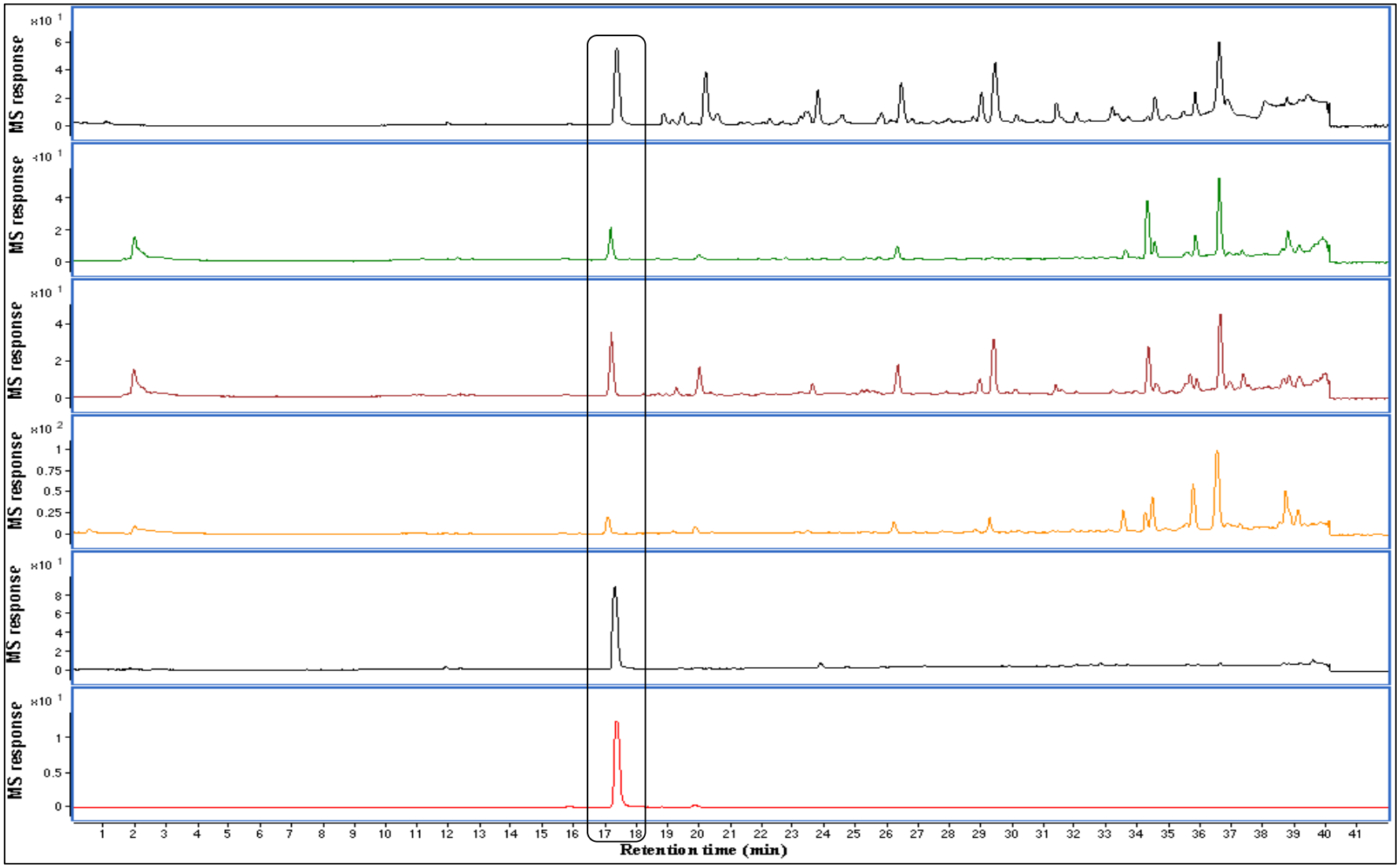

Figure 39. Identification of the schisandrol A in the plant extract YANG XIN® formulations and reference standard

In summary, we found that the majority of polar compounds like sibiricoses A5 were extracted in the methanol fraction because methanol is a more polar solvent and extracted the polar compounds in contrast to non-polar compounds like neoxanthin which partitioned into the dichloromethane extract because dichloromethane is a non-polar solvent. The use of ESI ion source enhance the ionization of polar compounds like ginsenoside Rf. The use of ESI for non-polar compounds such as β-Amyrin was a less efficient ionization strategy and APCI ionization gave better results. The use of positive mode was more favorable for compounds that have functional group that can accept proton like basic nitrogen atom in armepavine while the use of negative mode was more favorable for compounds with acidic functional group like the carboxylic acid group in poricoic acid A. The detection of some compounds reveals more than one peaks which could be isomeric forms of the proposed compound like in the case of batatasin III in figure 19 which shows two peaks that correspond to batatasin III and batatasin IV. The comparison of the extracted mass in figure 19 with batatasin III reference standard revealed that the first peak is batatasin III. Both ionization mode, positive and negative, detected the same number of analytical markers, nine analytical markers for each mode. The dichloromethane extract extracted 10 analytical markers in comparison to 8 analytical markers in the methanol extract. The use of APCI ion source was for ionization of non-polar compounds like β-amyrin, while more polar compounds was ionized very well with the use of ESI ion source.

2.3.3 Biological activity of plants in YANG XIN® formulations

Table 2 Biological activity of some plants included in YANG XIN® formulations

Markers	Biological activity	Reference
Angelica sinensis	Reduce sleep disorder	[6]
Cuscuta chinensis	Nourishing blood	[6]
Cistanche salsa	Prolong the duration of barbiturate-induced sleeping time	[6]
Astragalus membranaceus	Tonic effect and anti-oxidant	[17]
Rehmannia glutinosa	Treat cognitive impairment via enhancing endogenous antioxidant enzymatic activities and inhibiting free radical generation	[6]
Panax ginseng	Maintain balance in the body and help the body adapt to stress	[18]
Lycium barbarum	Increased energy level, stamina/endurance, sleep quality, ability to focus on activities, mental acuity, calmness, reduced feelings of fatigue, stress, headache, depression, daydreaming, impaired concentration, excess worry, unreasonable worry, memory loss	[19]
Biota orientalis	Sedative effect	[18]
Poria cocos	Anti-neurasthenia activity	[6]
Schisandra chinensis	Increase the body's capacity to work and support circulation	[18]
Polygala tenuifolia	Cognition improving effects	[6]
Zizyphus spinosa	Promote relaxation	[18]

Table 2 shows the biological activity of some of the plants included in YANG XIN® formulations.

Table 3 List of selected chemical markers and corresponding suppliers

Markers	Class	Purity (%)	Supplier	Catalog Number
Z-Butylidenephthalide	Phthalide	96	Sigma Aldrich	W333301-SAMPLE-K
4,5 Dicaffeoylquinic acid	Polyphenolic Compound	98	AvaChem Scientific	2086
Armepavine	Isoquinoline Alkaloid	95	Ryan Scientific Inc	STOCK1N-04340
Batatasin III	Stilbene	95	Analyticon Discovery	NP-012363
Cistanoside D	Phenylpropanoid Glycoside	95	Aapin Chemicals	20263 c
Formononetin	Isoflavone	Reagent Grade	Chromadex	ASB-00006191-050
Forsythoside A	Phenylpropanoid Glycoside	71	Analyticon Discovery	NP-003731
Ginsenoside Rf	Triterpenoid Saponin Glycoside	86	Chromadex	ASB-00007215-010
Neoxanthin	Tetraterpene	97	Bocsci	14660-91-4
Ophiopogonanone A	Homoisoflavonoid	98	A ChemtekInc	217-1330
Pinusolide	labdane-type diterpene lactone	98	A ChemtekInc	172-1204
Polygonatine A	Alkaloid	Not commercially available		
Poricoic acid A	Triterpene	98	ChemFaces	CFN92838
Schisandrol A	Lignan	98	Sigma Aldrich	SML0054-10MG
Sibiricoses A5	Sucrose ester	100	Analyticon Discovery	NP-018116
Swertisin	Flavonoid	78	Chromadex	ASB-00019441-010
β-Amyrin	Triterpene	99	Chromadex	ASB-00001745-010
α-asarone	Phenolic Ether	99	Chromadex	ASB-00011016-100

2.3.4 LC-MS chemical fingerprinting of YANG XIN® formulations

The same method for sample extraction described in Section 2.2.2.1 and sample preparation for plant fingerprinting described in Section 2.2.5 was used for the chemical fingerprinting of the YANG XIN® formulations, with the exception that a concentration of 0.75 mg/mL was used. LC-MS chemical fingerprinting of YANG XIN® formulations was achieved as shown in the following Figures 40-44. The visual inspection of the three different formulations in methanol and dichloromethane extracts in the positive and negative ionization modes revealed that YANG XIN® formulation seemed similar to the 2014-005_1B formulation and completely different from 2014-005_1A formulation.

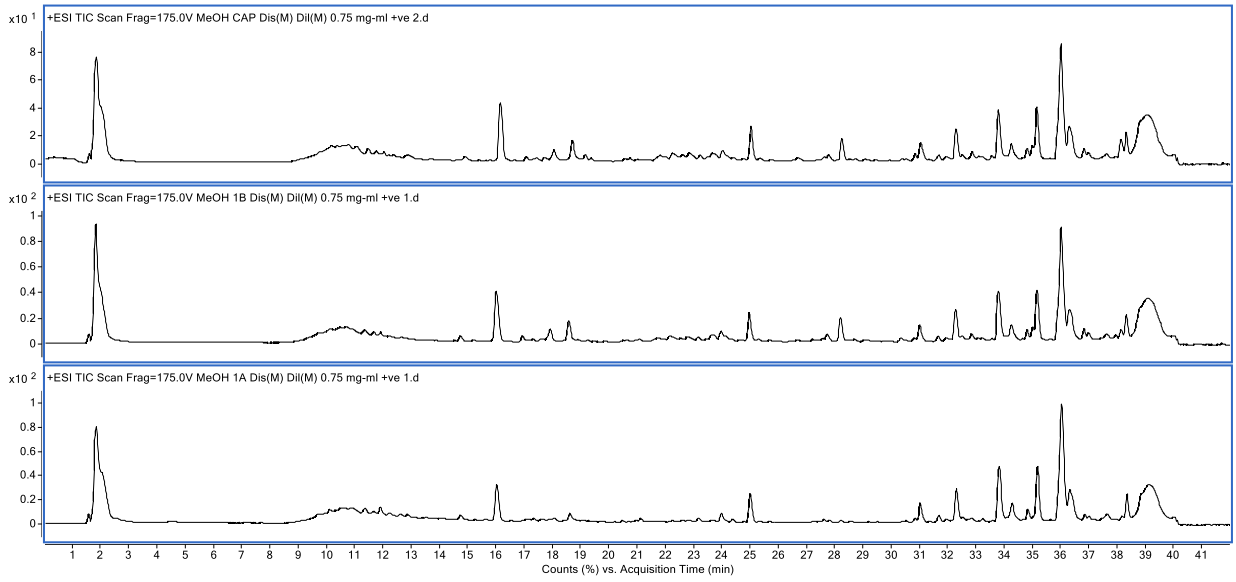

Figure 40. Total ion chromatogram of methanol extract of the three different formulations in positive mode

Type of extract: Methanol

Source of ionization: ESI

Ionization mode: Positive

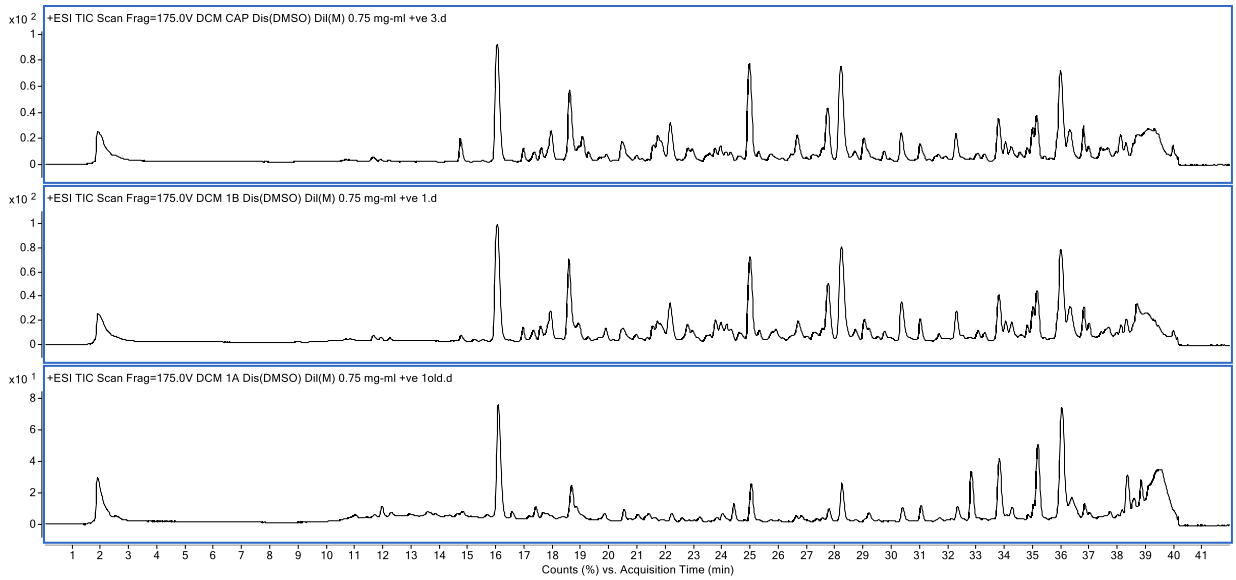

Figure 41. Total ion chromatogram of dichloromethane extract of the three different formulations in positive mode

Type of extract: Dichloromethane

Source of ionization: ESI

Ionization mode: Positive

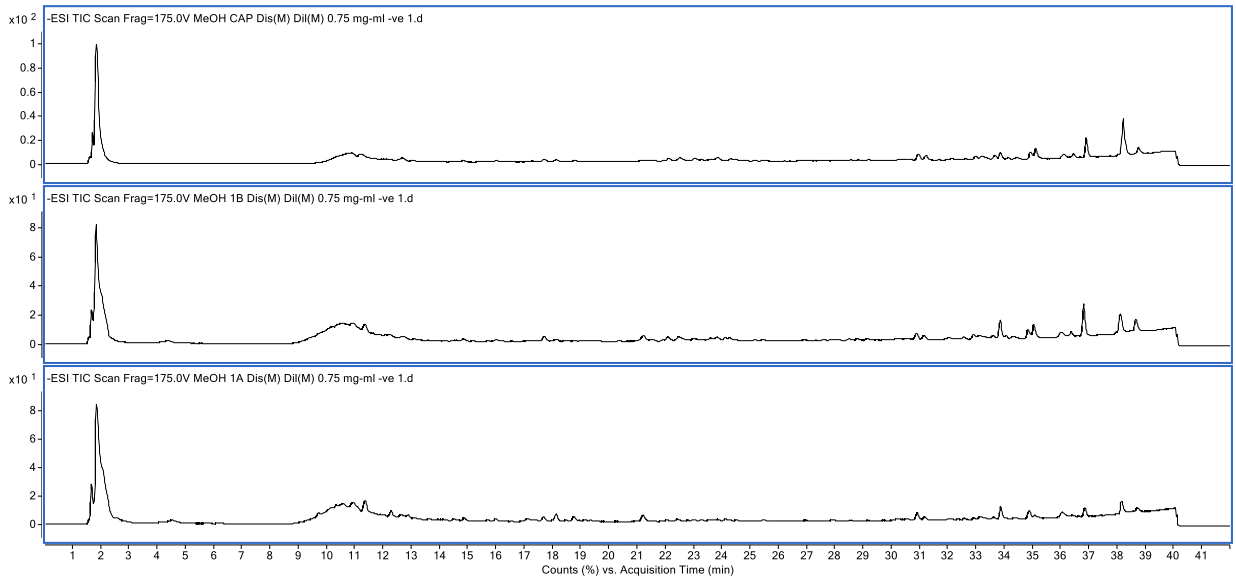

Figure 42. Total ion chromatogram of methanol extract of the three different formulations in negative mode

Type of extract: Methanol

Source of ionization: ESI

Ionization mode: Negative

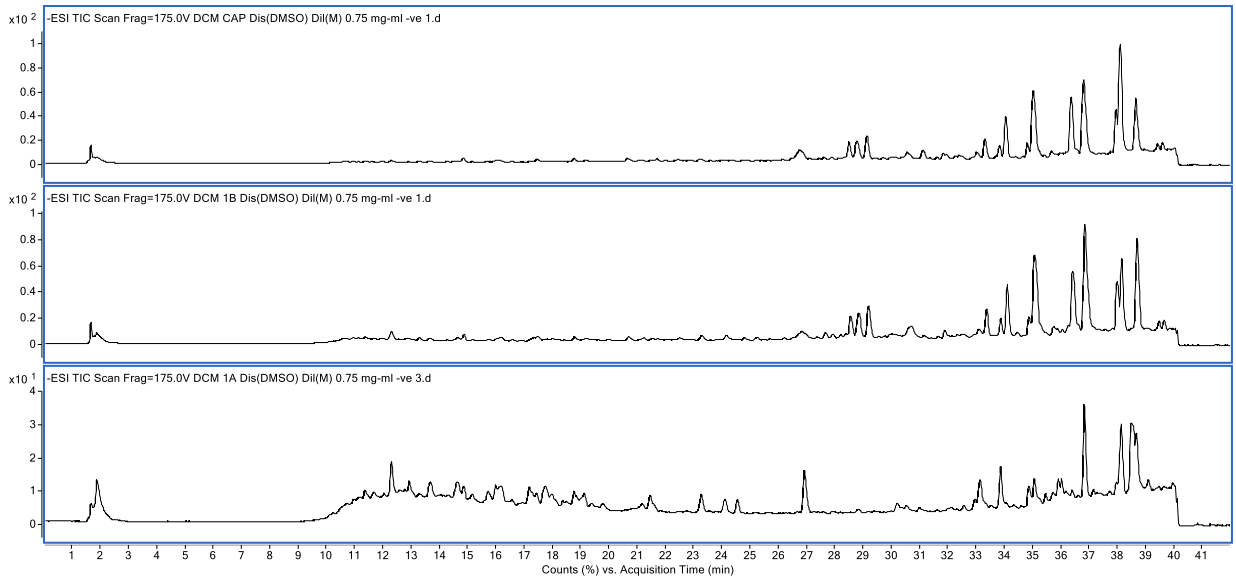

Figure 43. Total ion chromatogram of dichloromethane extract of the three different formulations in negative mode

Type of extract: Dichloromethane

Source of ionization: ESI

Ionization mode: Negative

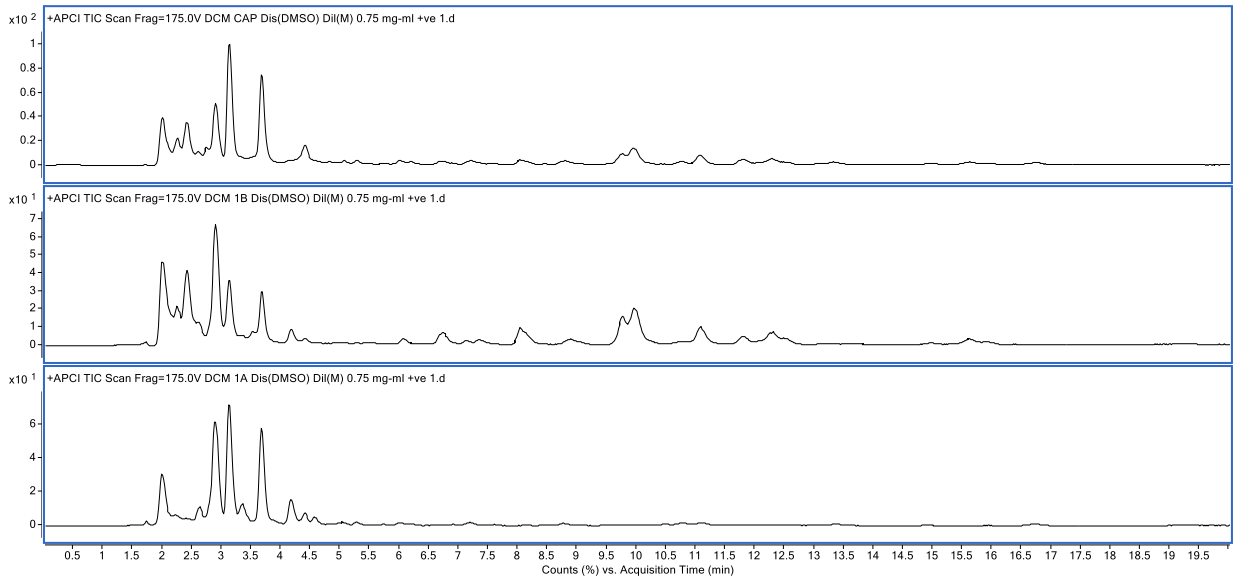

Figure 44. Total ion chromatogram of dichloromethane extract of the three different formulations in positive mode

Type of extract: Dichloromethane

Source of ionization: APCI

Ionization mode: Positive

2.3.5 ^{1}H NMR chemical fingerprinting of YANG XIN® formulations

Based on the ^{1}H NMR chemical fingerprinting method described in Section 2.2.4. ^{1}H NMR chemical fingerprints of YANG XIN® formulations were achieved as shown in the Figures 45-49. From the visual inspection of the NMR fingerprint of the methanol extract revealed that formulation 2014-005_1A showed higher relative intensity of signals in the region of δ_H 2.5 - 5.5 ppm in comparison to the formulation 2014-005_1B and YANG XIN® capsules. However, the relative intensity of chemical shifts in the ^{1}H NMR spectra of formulation 2014-005_1B and YANG XIN® capsules seemed to be identical. On the other hand, the ^{1}H NMR fingerprint of the dichloromethane extract of YANG XIN® formulation displayed higher relative intensity of the signals in the region of δ_H 0.6 − 6.8 ppm when compared with the formulation 2014-005_1B. One point worth discussing is that a ^{1}H NMR chemical fingerprinting of dichloromethane concentrated extract 2014-005_1A was not acquired due to very low amount of sample available for the NMR fingerprinting study.

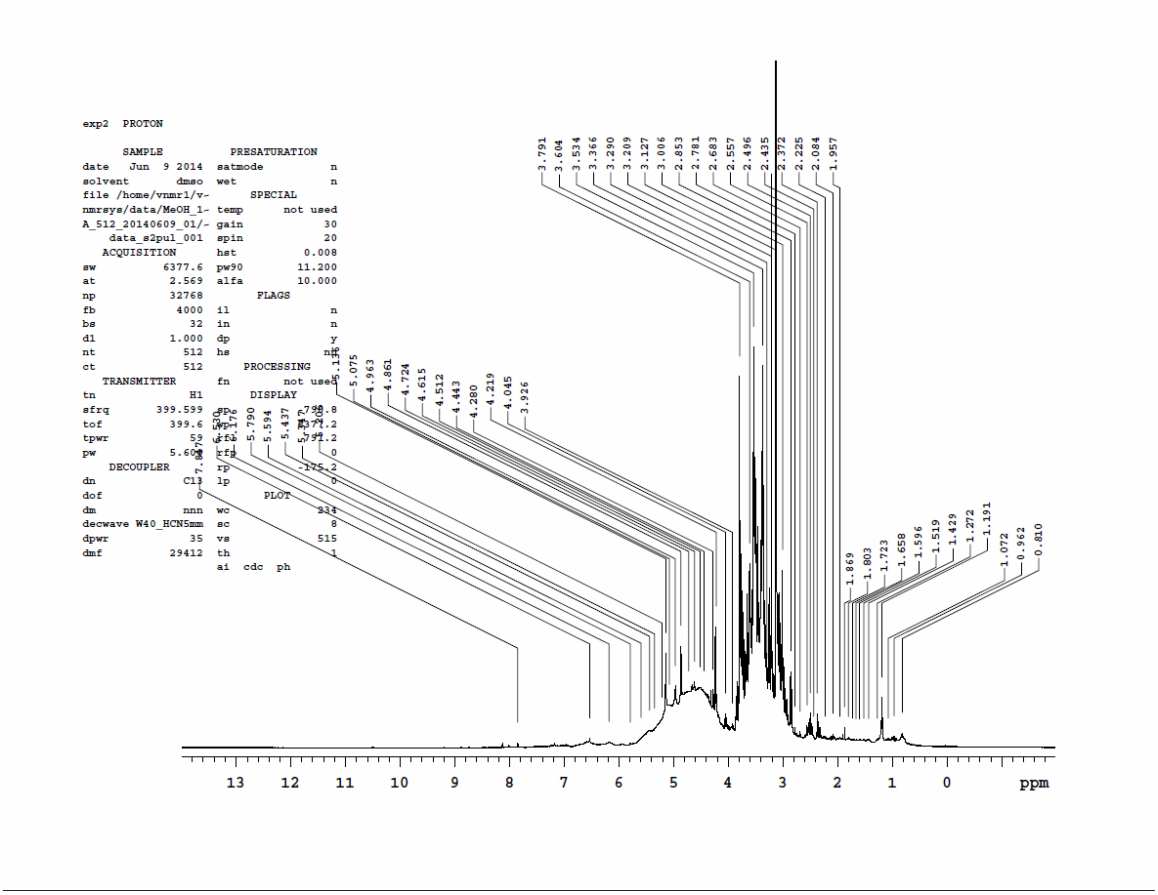

Figure 45. ^{1}H NMR fingerprint of methanol concentrated extract 2014-005_1A

Type of extract: Methanol

Solvent: DMSO- d_6

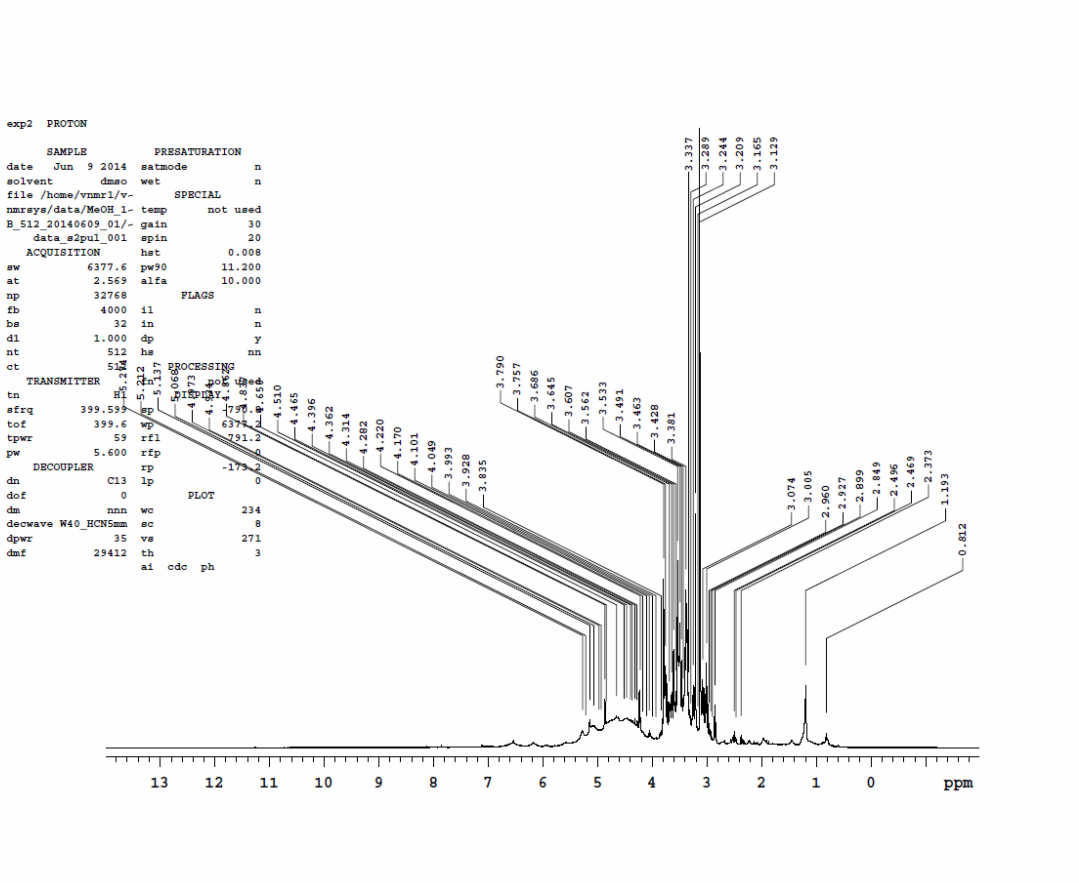

Figure 46. ^{1}H NMR fingerprint of methanol concentrated extract 2014-005_1B

Type of extract: Methanol

Solvent: DMSO- d_6

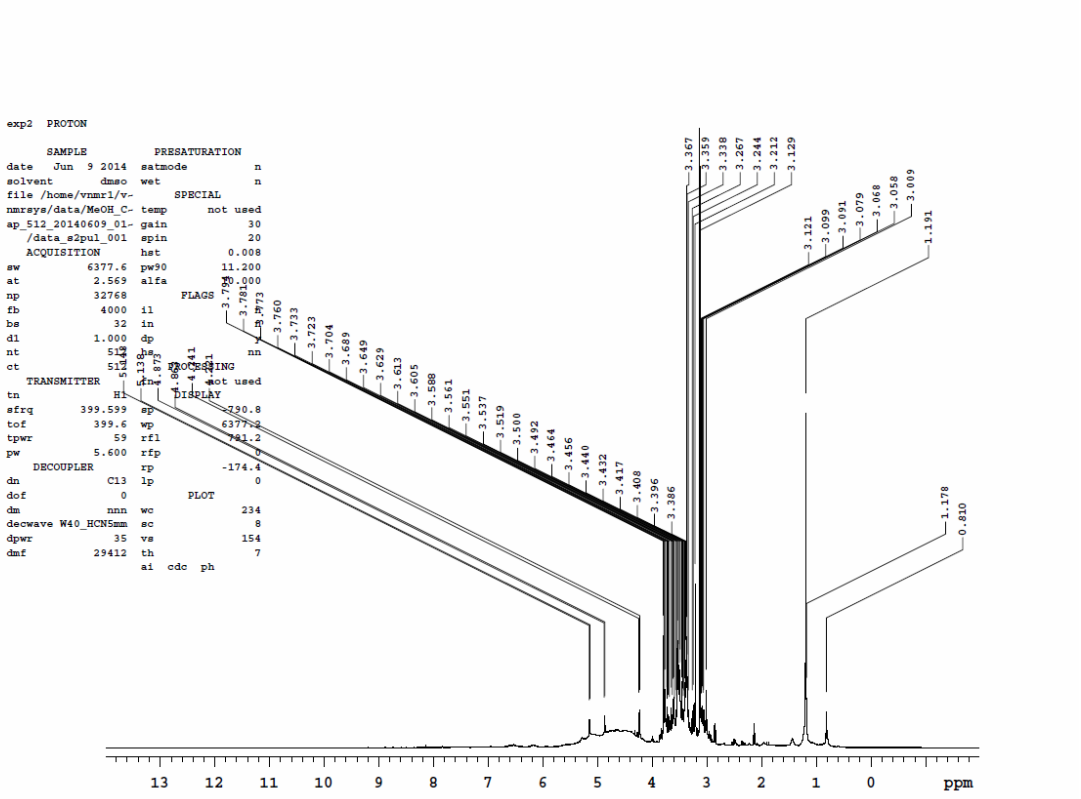

Figure 47. ^{1}H NMR fingerprint of methanol extract of YANG XIN® capsules

Type of extract: Methanol

Solvent: DMSO- d_6

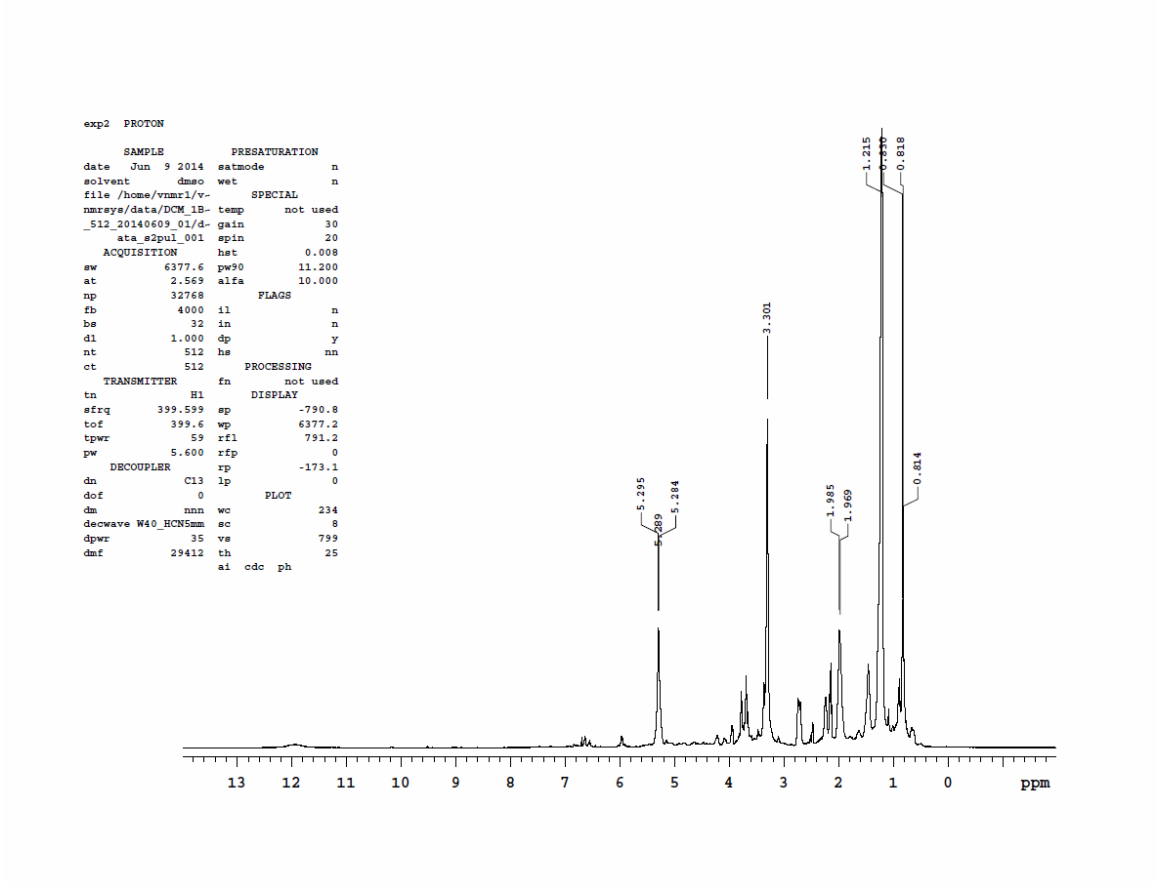

Figure 48. ^{1}H NMR fingerprint of dichloromethane concentrated extract 2014-005_1B

Type of extract: Dichloromethane

Solvent: DMSO- d_6

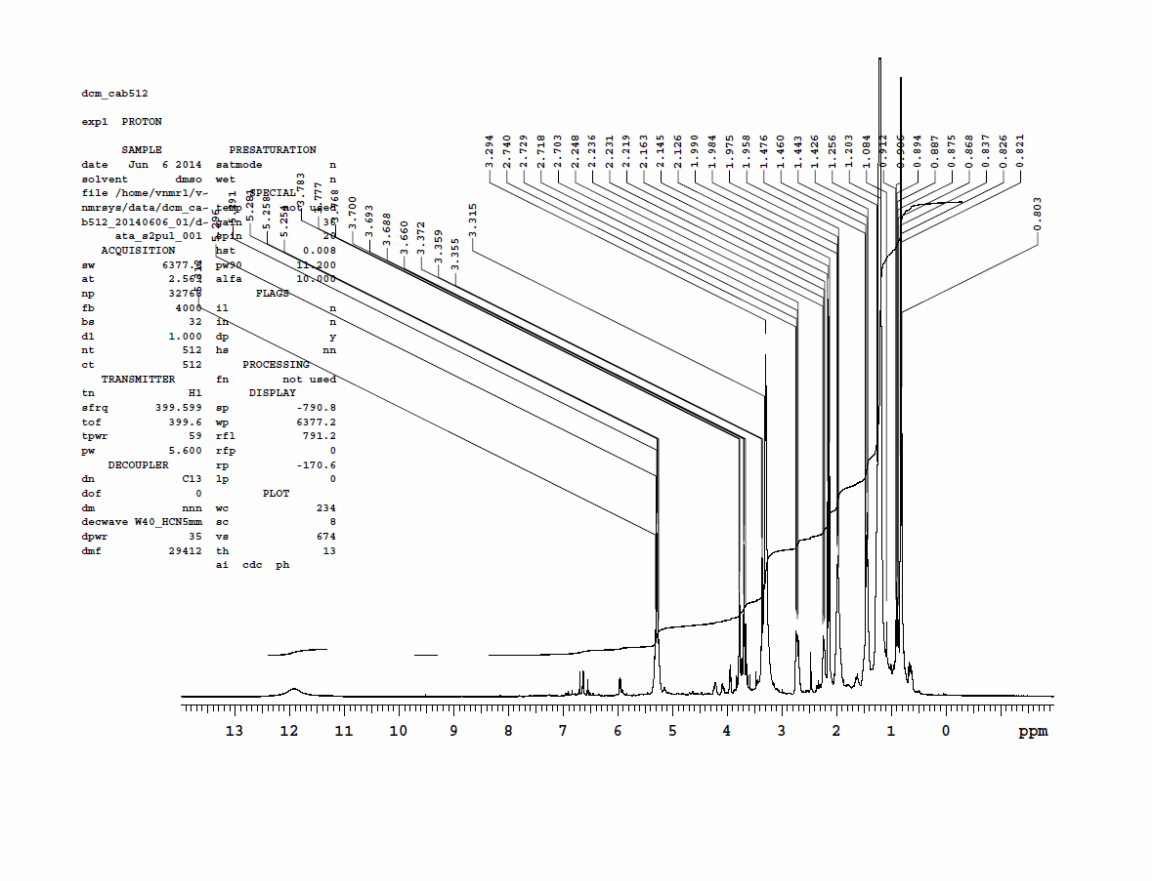

Figure 49. ^{1}H NMR fingerprint of dichloromethane extract of YANG XIN® capsules

Type of extract: Dichloromethane

Solvent: DMSO-d_6

2.4 Conclusion

In this section, we developed an optimal extraction procedure of YANG XIN® formulations twice with dichloromethane and methanol respectively and the LC-MS analysis of its chemical components. We selected 18 analytical markers for each plant present in the YANG XIN® formulation based on literature search on the compounds previously isolated from each plant ingredient. The identification of compounds relied on the data of accurate mass measurement, molecular formula and double bond equivalent (DBE), score, and error (ppm) of the measurement as well as the comparison of the data with the previously published data for the compounds. A comparison was made between the accurate mass of the proposed chemical marker with the corresponding accurate mass of chemical constituents of plant raw material included in the YANG XIN® formulation. Further confirmation of the selected chemical marker was done by matching of retention time, molecular formula, and double bond equivalent (DBE) of each chemical marker in the plant materials and extracts of YANG XIN® formulations, and the reference standard. Two ionization sources were used (ESI and APCI) in the LC-MS analysis. The use of LC-MS and ^{1}H NMR fingerprinting of the different formulations proved to be a useful technique to distinguish the three different formulations among each other and to assess consistency in the quality of such samples.

Chapter 3: Quantification of analytical markers

3.1 Introduction

After the qualitative analysis part in the previous chapter to identify analytical markers in the YANG XIN® formulation, gathering quantitative information is definitely a prerequisite for quality control. A considerable number of obstacles lie in the path of quantitative analysis of herbal medicine, not only because of its great diversity/variations in phytochemical components either because of species, origins and harvest season changes [20,21], or presence of some toxic compounds like in the plants of the genus *Digitalis* [10], but also due to the lack of authentic reference standard [22]. According to a review paper written by Li *et al* in 2011, only 400 reference compounds were available for TCM products. This low number of reference standards might be due to instability of reference compounds or the challenging isolation of trace amounts from plants. About 1.5 billion people are using TCM preparations worldwide [20]. Therefore, quality control of TCM products is very important to ensure safety and efficacy since they might contain chemical adulterants and toxic compounds. Also, TCM products must meet the criteria of many governmental regulatory agencies such as FDA and EMA that clearly state that proper fingerprinting should be used to assess the consistency of botanical products [21]. Herbal identification and authentication by the WHO and Chinese pharmacopeia commission are also required [12].

TCM formulations are usually made from a combination of many plants/herbs, which contain hundreds or even thousands of different chemical compounds. Moreover, usually the compound of interest may be present in a very low concentration (<100 μg/g) [23]. In order to quantify each plant to ensure safety and efficacy [20], a so-called marker compound for each plant

is selected for the purpose of quantitative analysis. Quantification of a small amount of a natural product in a complex mixture requires state of the art analytical methods [12]. Using a hyphenated technique such as LC coupled to mass spectrometry is considered the best choice and has been extensively applied for quality control of TCM products [20]. LC is the most popular instrument for chromatography because of ease of use and wide suitability [24] and coupling to an MS detector that provides higher selectivity and sensitivity for assaying trace amounts of compounds [25]. These features of LC-MS makes the quantification of trace amount natural products' metabolite easier within a few minutes with high level of sensitivity [12].

3.2 Materials and methods

3.2.1 Chemicals

LC-MS solvents, reference compounds, and formulations used were described previously in detail in the Section 2.2.1.

3.2.2 Preparation of standard solutions

Each reference standard was dissolved in methanol in a concentration of 5 mg/mL except for neoxanthin 5 mg/mL in DMSO and formononetin 2.5 mg/ml in methanol then diluted for a series of dilution according to dilution factor 1:2. Samples were kept in -80 °C freezer until the date of the analysis.

3.2.3 Calibration curves

Working solutions with concentrations of 0.0076, 0.0153, 0.0305, 0.0610, 0.1221, 0.2441, 0.4883, 0.9766, 1.9531, 3.9063, 7.8125, 15.6250, 31.25, 62.50 µg/mL were prepared by diluting the stock solutions of reference standards with methanol. Then each working solution was

analyzed using LC-MS for the preparation of calibration curves and the determination of linearity, limit of detection (LOD) and limit of quantitation (LOQ).

3.2.4 Linearity and detection limits

The linearity of the calibration curves were constructed using 15 different concentrations of the selected chemical markers utilizing a least square linear regression method. Each concentration was injected three times. Reference standard calibration curves were constructed by plotting the peak area ratio of each standard against the expected concentrations of the calibration reference standards. Various weighting factors were used depending on the chemical marker. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the concentrations with signal-to-noise ratios of 3 and 10, respectively. The linear range was investigated up to an analyte concentration of 62.50 µg/ml. To evaluate the method's precision and accuracy, the intra-day variation for the mixed reference standard solution was analyzed 9 times using 3 different concentrations in 1 day to determine the intra-day variation, and on 3 consecutive days to determine the inter-day variation using calibration curves that were prepared previously. Relative standard deviation and relative errors were expressed for both intra-day and inter-day variation respectively.

3.2.5 Recovery

In order to assess the recovery of the extraction method, we spiked ophiopogonanone A, one of the selected chemical markers for YANG XIN® formulations at a concentration 0.2441 µg/mL into the YANG XIN® capsules then extracted as mentioned before in the extraction method. Three replicate injections were performed in the recovery study.

3.3 Results and discussion

3.3.1 Quantitation of chemical markers in YANG XIN® formulations

A method based on LC-MS for the quantitative analysis of 17 chemical markers in Yang Xin® capsules, and two concentrated extracts 2014-005_1A and 2014-005_1B has been developed. The utility of our new method was demonstrated in the quantitative analysis of the first 17 chemical markers in the three formulation samples (Tables 4, 5 & 6). No internal standard was used for the quantitation of YANG XIN® formulations because of the lack of isotopic labeled compounds on the market for each chemical markers selected for YANG XIN® formulations. Based on the obtained results from Tables 4, 5 and 6, YANG XIN® capsules and 2014-005_1B formulation appear to be similar to each other and different from 2014-005_1A formulation based on the fact that both YANG XIN® capsule and 2014-005_1B formulations contain same analytical markers. Fifteen chemical makers were identified and quantified in comparison to 10 markers in 2014-005_1A formulation. The formulation 2014-005_1B contains the highest amount of chemical markers followed by the formulation YANG XIN® capsules then 2014-005_1A. Good linearity correlation response was observed for the different analytical markers (r^2= 0.9945 – 0.999). The R.S.D of the intraday precisions were in the range (0.4 -3.9%) with accuracy (99.2 – 101%) whereas the R.S.D of interday precisions were within (0.5 - 5.6%) with accuracy (99.6 – 100.2%). The limits of detection and quantitation were (0.0011 – 0.0732 µg/mL), and (0.0038 – 0.2441 µg/mL) respectively. The results suggest the ability of this method to detect the 17 analytical markers of YANG XIN® formulations with high sensitivity and selectivity.

3.3.2 Recovery

In order to assess the recovery of the extraction method, ophiopogonanone A, one of the 18 markers, was selected as a candidate for this test due to two reasons. First, it displayed LOQ value in the middle concentration range when compared with other markers and second it showed the same LOQ values as other (6) markers from this study. The reference standard was spiked at a concentration of 0.2441 µg/mL into the YANG XIN® capsules then extracted as mentioned before in the extraction method. Three replicate injections were performed. The results showed recovery of 145. 7% with R.S.D value of 0.2 %. A further recovery study could be done in the future to assess the extraction efficacy of the remaining markers.

Table 4 Linear regression equation analysis in the determination of the 18 analytical markers

Compound	t_R(min)	Ions (*m*/*z*)		Linear range (μg/ml)	Weighting factor	Slope (*a*)	Intercept (*b*)	r^2 (*n* = 3)	LOD (μg/ml)	LOQ (μg/ml)
		(+) ESI $[M+H]^+$	(-) ESI $[M-H]^-$							
Z-Butylidenephthalide	19.91	189.0915		0.0305 – 3.9063	1/X	2681159.9783	26669.1011	0.9986	0.0092	0.0305
4,5 Dicaffeoylquinic acid	11.22		515.1167	0.0038 – 3.9063	1/X	841803.4303	1181.1532	0.9984	0.0011	0.0038
Armepavine	10.00	314.1783		0.0038 – 0.1221	None	46799070.3241	184499.2967	0.9951	0.0011	0.0038
Batatisin III	14.50		243.0992	0.0038 - 0.2441	None	1161644.9241	9665.6863	0.9978	0.0011	0.0038
Cistanoside D	11.58		651.2237	0.0038 – 0.2441	None	332783.4634	2339.2292	0.9977	0.0011	0.0038
Formononetin	16.98		267.0655	0.0076 - 3.9063	1/X	6920982.1854	14282.9095	0.9966	0.0023	0.0076
Forsythoside A	10.69		623.1993	0.0076 – 15.625	1/X	1458343.0021	7743.7846	0.9990	0.0023	0.0076
GinsenosideRf	18.53	423.3620$^+$		0.0076 - 3.9063	1/X	879855.6470	1282.2609	0.9993	0.0023	0.0076
Neoxanthin	36.80	601.4251		0.2441 – 7.8125	None	104558.0013	41502.8582	0.9949	0.0732	0.2441
Ophiopogonanone A	23.78		327.0900	0.0076 - 3.9063	None	6872049.7566	9150.0624	0.9999	0.0023	0.0076
Pinusolide	23.70	347.2216		0.0038 – 0.2441	1/X	4407492.7747	13477.1997	0.9961	0.0011	0.0038
Polygonatine A	9.60	166.0861		-	-	-	-	-	-	-
Poricoic Acid A	30.99		497.3300	0.0076 - 3.9063	None	5995277.1332	7786.0208	0.9990	0.0023	0.0076
Schisandrol A	17.30	433.2250		0.0153 – 3.9063	1/X	3107568.1846	12478.8984	0.9960	0.0046	0.0153
Sibiricoses A5	10.33		517.1580	0.0153 - 3.9063	1/X	800365.3913	-9172.8189	0.9990	0.0046	0.0153
Swertisin	11.45		445.1200	0.0076 - 3.9063	None	1782135.5123	949.7725	0.9998	0.0023	0.0076
β-Amyrin	10.71	409.3800*		0.0153 – 0.4883	None	482048.0949	14866.2519	0.9945	0.0046	0.0153
α-Asarone	18.32	209.1130		0.0153 – 3.9063	1/X	3537199.5011	32358.3206	0.9979	0.0046	0.0153

+: $[M+H - GlcGlc - H_2O]^+$; *: $[M+H-H2O]^+$

Table 5 Contents (μg/g) of the 18 analytical markers in the 3 samples (n = 3)

Chemical Markers	Content of each compound in 18 samples (μg/g)[A]		
	2014-005_1A	2014-005_1B	Capsules
Z-Butylidenephthalide	0.00 ± 0.00 *	24.61 ± 0.34	28.36 ± 0.83
4,5 Dicaffeoylquinic acid	13.26 ± 5.43	15.67 ± 0.28	8.13 ± 0.00 †
Armepavine	0.00 ± 0.00 *	0.00 ± 0.00 *	0.00 ± 0.00 *
Batatisin III	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Cistanoside D	0.08 ± 0.01	6.82 ± 0.24	4.46 ± 0.11
Formononetin	16.38 ± 0.27	6.33 ± 0.09	6.28 ± 0.11
Forsythoside A	131.72 ± 3.49	107.53 ± 2.10	449.25 ± 9.84
GinsenosideRf	107.39 ± 2.44	47.03 ± 1.62	2.10 ± 0.00 †
Neoxanthin	0.00 ± 0.00 *	338.05 ± 14.15	88.56 ± 8.15
Ophiopogonanone A	0.00 ± 0.00 *	0.02 ± 0.01 *	0.03 ± 0.00 *
Pinusolide	0.00 ± 0.00 *	0.09 ± 0.10 *	0.06 ± 0.06 *
Polygonatine A	N.D.	N.D.	N.D.
Poricoic Acid A	0.01± 0.00	6.58 ± 0.21	1.66 ± 0.12
Schisandrol A	8.40 ± 0.10	933.09 ± 21.85 †	575.77 ± 15.12
Sibiricoses A5	250.45 ± 4.81	251.91 ± 7.44	245.58 ± 12.96
Swertisin	3.77 ± 1.25 †	10.19 ± 0.81	18.83 ± 0.92
β-Amyrin	0.20 ± 0.00	2.32 ± 0.41	1.03 ± 0.38
α-Asarone	0.00 ± 0.00 *	168.99 ± 6.36	163.69 ± 1.96

A: Mean ± Std. Dev.; †: n < 3; *: below quantitation level; N.D: Not determined

Table 6 Statistical results of precision and accuracy of the 18 markers

Compound	Intra-day		Inter-day	
	Accuracy (R.E., %)	Precision (R.S.D., %)	Accuracy (R.E., %)	Precision (R.S.D., %)
Z-Butylidenephthalide	99.8	1	99.6	1.1
4,5 Dicaffeoylquinic acid	100.3	2.3	100.2	3.9
Armepavine	99.7	1	99.6	1.1
Batatisin III	100	0.8	100.1	1.5
Cistanoside D	100	2.6	100.2	3.7
Formononetin	99.8	0.4	100	0.5
Forsythoside A	100.1	0.8	100	1.2
GinsenosideRf	99.9	1.6	99.8	1.8
Neoxanthin	99.2	3.9	99.9	5.6
Ophiopogonanone A	100.2	1	100.2	1.1
Pinusolide	99.9	1.2	99.9	1.5
Polygonatine A	N.D.	N.D	N.D.	N.D.
Poricoic Acid A	101	3.5	100	3.8
Schisandrol A	100	0.7	99.8	0.9
Sibiricoses A5	99.9	0.8	100	1.2
Swertisin	100.2	0.7	100	1.2
β-Amyrin	99.9	1.4	99.9	1.8
α-Asarone	99.7	0.8	99.6	1

N.D: Not determined

3.4 Conclusion

In this work, we were able to quantify 17 markers that represent 17 plants in the different YANG XIN® formulations for the first time. The YANG XIN® capsules and 2014-005_1B formulation were similar to each other and different from 2014-005_1A formulation based on the fact that both YANG XIN® capsule and 2014-005_1B formulations contain same analytical markers. This method provides good linearity (r^2> 0.9945), intraday precisions (R.S.D< 3.9%), interday precisions (R.S.D< 5.6%), accuracy (99.2 - 101%), recovery (145.7%), limit of detection (0.0011 – 0.0732 µg/mL), and limit of quantitation (0.0038 – 0.2441 µg/mL). The newly developed method proved to be a good tool for the quality assessment of YANG XIN® formulations with sensitivity and selectivity

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