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약학석사 학위논문

Metabolomics Study in *Cnidium officinale* Accessions by Multivariate Analysis

다변량 분석 기법을 활용한 천궁 계통의 대사체학 연구

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Abstract

Cnidium officinale is a plant belonging to the genus Umbelliferae. Its rhizome, Cnidii Rhizoma, has traditionally been used for many purposes such as blood circulation and analgesic effects, and has recently been reported to have anti-inflammatory activity and significant efficacy in female diseases. In Korean Pharmacopoeia, Cnidii Rhizoma has designated rhizome of both *Cnidium officinale* and *Ligusticum chuanxiong*. Although *C.officinale* and *L. chuanxiong* are the official source for Cnidii Rhizoma in Korea, sometimes they are intentionally or non-intentionally adulterated with the root stem of *Ligusticum sinense* due to their morphological and chemical similarities. In addition, *C. officinale* and *L. chuanxiong* are designated to the origin plants of Cnidii Rhizoma in the Japanese pharmacopoeia (JP) and the Chinese pharmacopoeia (CP), respectively. With the rise of international trade, increasingly, it is unclear which plant is used for Cnidii Rhizoma. Thus, it is important to clearly discriminate the origin of Cnidii Rhizoma.

In the present study, we investigated the metabolic differences among genera or species for Cnidii Rhizoma using UPLC-DAD-QTOF-MS combined with multivariate analysis. The spectral data of *C. officinale*, *L. chuanxiong* and *L. sinense* derived from UPLC-DAD-QTOF-MS or NIRS were analyzed by principal component analysis (PCA) to evaluate their clustering pattern. When there is a clustering pattern between the samples, an OPLS-DA model was constructed based on UPLC-MS data and the metabolic markers contributing the clustering pattern were derived through S-plot analysis. As a result, senkyunolide I and senkynolide H were detected only in *L. sinense* whereas bisabolangelone was not detected only in the same sample, when comparing the MS fragment sepctra and DAD spectra of the tested samples.

Since *C. officinale* and *L. chuanxiong* were almost identical in chemical composition, we tried to identify the important metabolites most contributing to distinguish them from each community using PCA and S-plot of OPLS-DA of their MS data. The results of this analysis showed that the content of phthalide type metabolites including *Z*-ligustilide and diligustilide leads to a meaningful distinction. Among them, the content of diligustilide type compounds were low in the plants and their UV absorbance was consequently low either. Therefore, the quantitative determination method for *Z*-ligustilide was developed using HPLC-DAD. In addition, the NIR spectra of the tested samples and their Z-ligustilide contents were compared.

Taken together, UPLC-DAD-QTOF-MS analysis and the multivariate analysis of the spectral data of the *C. officinale* accessions could find their metabolic differences . Therefore, this study can be a reference for the differentiation *of C. officinale, L. chuanxiong* and *L. sinense.* Moreover, a rapid determination method for some important metabolic markers, Z-ligustilde, was developed using NIR spectroscopy, which was confirmed by HPLC-DAD.

Keywords : *Cnidium officinale*, *Ligusticum chuanxiong*, *Ligusticum sinense*, Cnidium Rhizome, UPLC-QTOF-MS, HPLC-UV, NIRS, metabolomics, multivariate analysis, PCA, PLS-DA, OPLS-DA

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List of Abbreviations

AcCN : acetonitrile

ESI : electron spray ionization

MeOH : methanol

EtOH : ethanol

HPLC : high performance liquid chromatography

LC : liquid chromatography

MS : mass spectrometry

NIRS : near-infrared spectroscopy

OPLS-DA : orthogonal projections to latent structure-discriminant analysis

PCA : principal component analysis

QC : quality control

QTOF : quadrupole time of flight

R_t : retention time (RT)

UPLC : ultra performance liquid chromatography

VIP : variable importance in projection

UV: ultraviolet absorption spectroscopy

I. Introduction

Cnidium Rhizome (Umbelliferae)

Cnidium Rhizome is a member of the Umbelliferae family, which uses roots as medicinal sites and has been traditionally used for the treatment of a variety of diseases, including promote blood circulation and analgesic. Recently, anti-inflammatory and gynecological disease treatment activities have been reported (Jeong et al., 2012, Choi et al., 2011). Cnidium Rhizome, in addition to its own herbal medicines, interest in the efficacy of Cnidium Rhizome-derived substances has increased, and studies on the components in the Cnidium Rhizome have been actively conducted. Among them, various component studies have been conducted on roots used as medicinal sites (Li et al., 2002, Li et al., 2003, Yi et al., 2006). Several major phthalic anhydride derivatives such as ligustilide, senkyunolide A, and senkyunolide I and phenylpropanoid compounds such as ferulic acid, chlorogenic acid, and coniferyl ferulate have been reported.

However, for each pharmacopeia in the countries, the origin plant for Cnidium Rhizome is different. In KP is the origin of the plant, designated as both *Cnidium officinale* Makino and *Ligusticum chuanxiong* Hort. But in JP, only *C. officinale* is designated as the original plant, while in CP, only *L. chuanxiong* is designated as the original plant. Also in Korea Food Standards Codex, only *C. officinale* is designated as the origin plant. Thus, the need for differentiation of these plants arises because the origin plants for Cnidium Rhizome are different for each country and pharmacopoeia. In addition, *L. sinense*, which is similar in appearance and has similar components, is negatively incorporated as a substitute for Cnidium Rhizome because its value is relatively inexpensive. Therefore, *L. sinense* also needs to be distinguished (Figure 1).

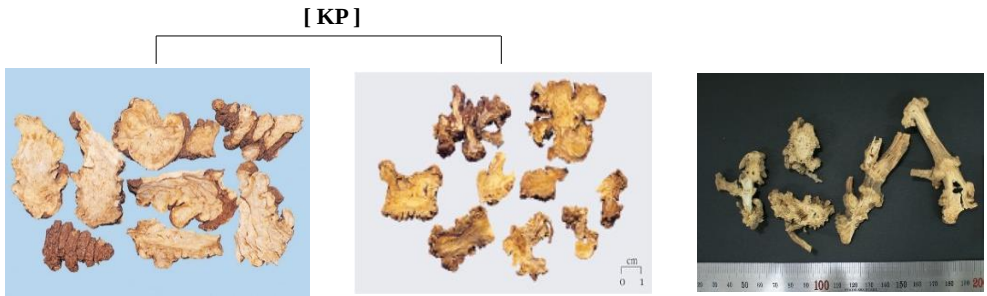


Figure 1. *C. officinale*, *L. chuanxiong* and *L. sinense* in pharmacopoeia

Genetic differentiation of *C. officinale* accessions

Identification of accurate herbal medicines and identification of herbaceous plant species is recognized as a very important factor in Chinese medicine standardization. The sensory evaluation criteria are morphological changes occurring in different growth environments of plants, and it is difficult to discriminate correctly if there are species with similar shape in the same genus. From 1996 to 2015, studies related to the origin of *C. officinale* accession plants have been carried out steadily genetically (Kondo et al., 1996, Song et al., 2009, Han et al., 2015). In these papers, *C. officinale* is said to form similar gradation to *Ligusticum* genus rather than *Cnidium* genus in strict consensus tree of Apioideae resulting from phylogenetic analysis of rbcL sequences (Figure 2).

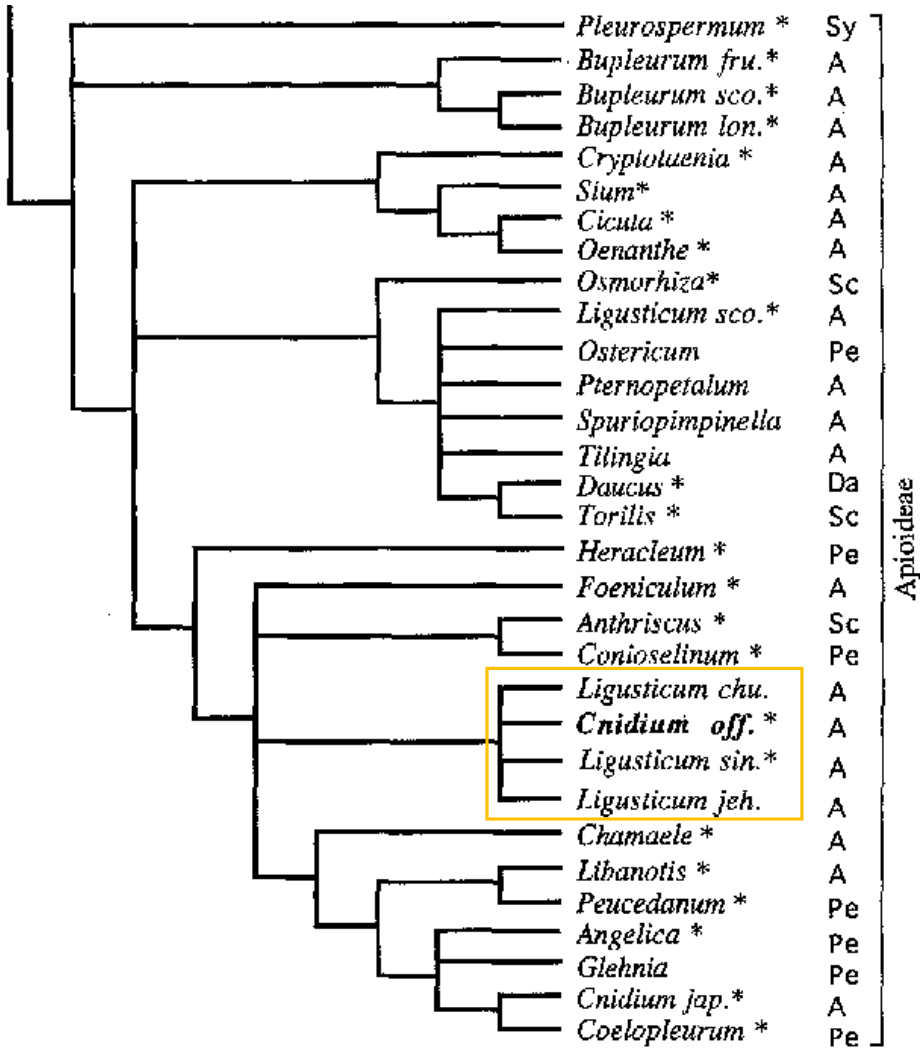


Figure 2. Strict consensus tree of Apioideae resulting from phylogenetic analysis of rbcL sequences

Moreover, strict consensus tree of Apioideae resulting from phylogenetic analysis of the reduced matrix shows that *C. officinale* and *L. sinense* are very similar in gradation (Figure 3).

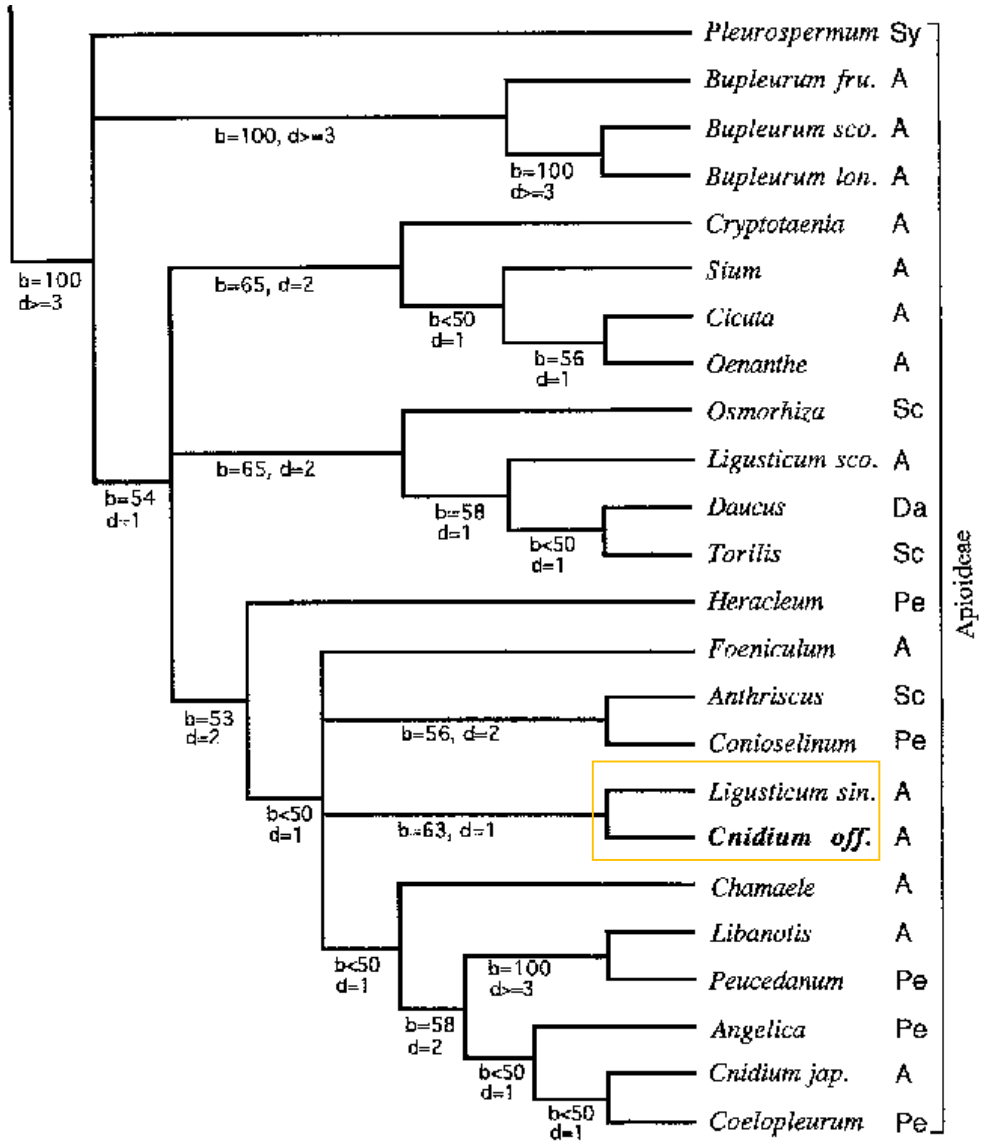


Figure 3. Strict consensus tree of Apioideae resulting from phylogenetic analysis of the reduced matrix

And the research says that *C. officinale* and *L. chuanxiong* have the same sequences, and there is only one base substitution between *C. officinale* and *L. sinense*. To distinguish *C. officinale* and *L. chuanxiong*, molecular markers for the identification of astrocytes have also been studied. It is known that there are single

nucleotide polymorphisms (SNPs) that can be distinguished between two plants at one or two positions.

Physicochemical differentiation of *C. officinale* accessions

In this study, the metabolites of *C. officinale* accessions were investigated from the viewpoint of metabolomics. Metabolomics is used in a wide range of fields, including biology, plant physiology research, and quality control of crops. Metabolomics in medicinal plants can be an effective tool for metabolism analysis and profiling. Typical equipment includes spectroscopic techniques such as liquid or gas chromatography coupled with mass spectrometry, which are used primarily for quality control of medicinal plants (Sawada et al., 2009, Lee et al., 2012).

Numerous hyphenated techniques have been applied to natural materials analysis, including LC-MS, GC-MS, HPLC-ESI-MS / MS and UPLC- (Q) TOF-MS (/ MS). Of these, UPLC-Q-TOF-MS (/ MS) is the most advanced technology for rapid chemical profiling of medicinal plants. UPLC improves the retention time reproducibly and increases resolution and sensitivity. UPLC-Q-TOF-MS (/ MS) can be used for a variety of plants including the ciliate, since Q-TOF-MS has low energy collision-induced dissociation and provides an accurate mass value of the analyte. It is one of the most widely used methods for analyzing secondary metabolites. Currently, the quality standard of *C. officinale* accessions is based on the content of the marker ferulic acid and ligustilide which are bioactive compounds identified in (Li et al., 2003, Ji et al., 1999, Lu et al., 2004). In order to determine the quality of the natural products, it is important to have a fast and accurate method for the analysis of the standard components.

Therefore, metabolite studies were carried out on three kinds of plants which are *C. officinale, L. chuanxiong* and *L. sinense*. Total of 36 samples were subjected

to secondary metabolite profiling based on UPLC-MS. Based on the UPLC-MS and NIRS, multivariate analysis was performed to determine whether there is a distinction between the accessions according to the metabolites, and to identify markers that greatly affect the classification.

Phthalide anhydride compounds

Several types of compounds have been isolated and identified including phenols, alkaloids and phthalides. Various known phthalide anhydride compounds in *C. officinale* accessions have been reported in *Ligusticum* as essential oil components (Li et al., 2002, Li et al., 2003, Yi et al., 2006, Pushan et al., 1984, Kobayashi et. al., 1987). Phthalide is a type of lactone that is used as the core chemical structure of more complex chemical compounds including dyes, fungicides and natural oils. These phthalide-based compounds are used in the synthesis of several medicines such as angiogenesis inhibitor, antihypertension and insecticide. Phthalide type compounds (Figure 4) are, mostly in liquid form, and exhibit fairly low stability. Therefore, storage conditions and solvent selection are important.

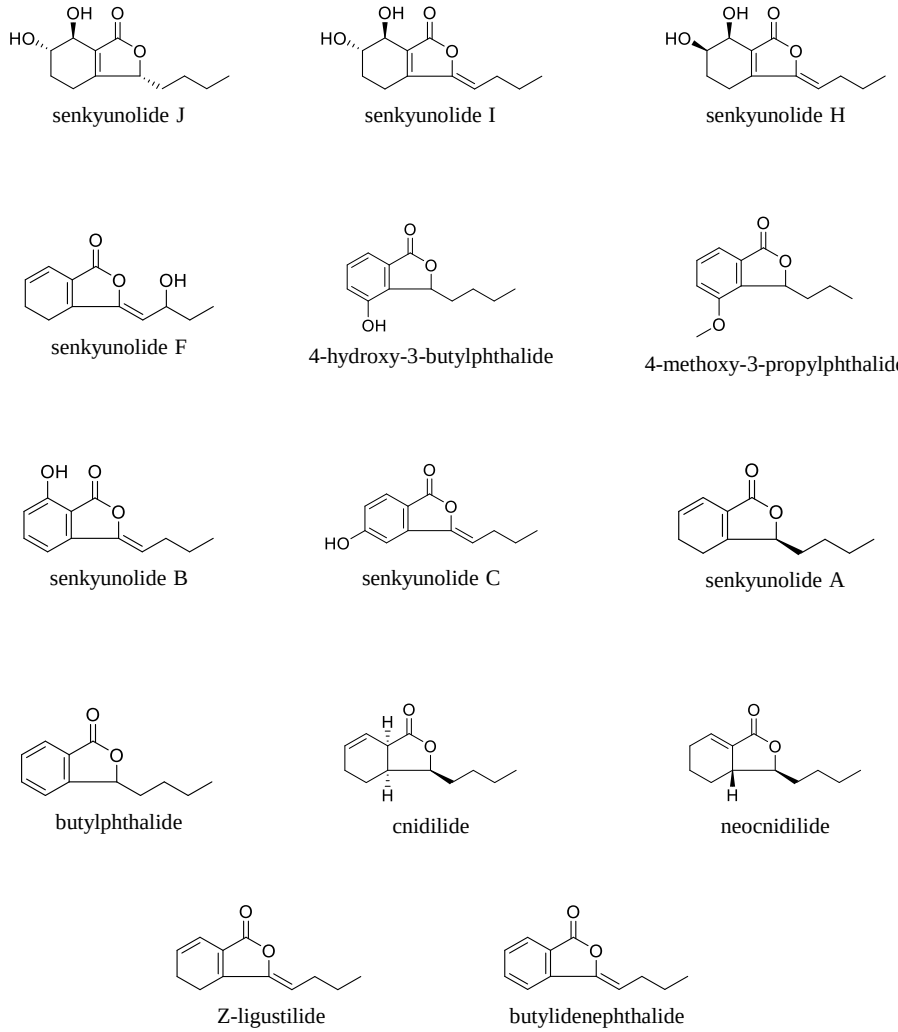


Figure 4. Structures of phthalide type compounds

II. Secondary metabolite profiling in *C. officinale* accessions

A. Materials and methods

1. Materials

Total thirty six of *C. officinale* accession samples were analyzed. Among the samples, twenty four roots of *C. officinale,* eleven roots of *L. chuanxiong* and one root of *L. sinense* were used (Table 1). In case of *C. officinale*, twenty samples were domestic and four samples were from China. In addition, every sample of *L. chuanxiong* and *L. sinense* were collected in Korea. All of them were imported from different herbal markets and forest medicinal resources research institutes from different countries (Table 2).

2. Reagents and instruments

2.1. Reagents

2.1.1. Reagents for UPLC-MS analysis

The acetonitrile, methanol, ethanol and water used in the extraction of the experimental material, the sample preparation and the UPLC analysis were analyzed by J.T. HPLC grade solvent (Baker®, Avantor Performance Materials, Inc., USA). And formic acid for mass spectrometry was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.1.1. Reagents for HPLC-DAD analysis

The acetonitrile, methanol, ethanol and water used in the extraction of the experimental material, the sample preparation and the HPLC analysis were analyzed by J.T. HPLC grade solvent (Baker®, Avantor Performance Materials, Inc., USA). And formic acid for mass spectrometry was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Instruments

UPLC-QTOF MS system

- Waters Acquity UPLC® system, Waters Co., Milford, MA, USA
- Column : Waters Acquity UPLC BEH C18, 2.1 mm x 100 mm, 1.7 μm
- Waters Xevo G2 QTOF mass spectrometer, Waters MS Technologies, Manchester, UK

HPLC-DAD system

- Thermo Ultimate 3000 (Thermo Fisher Scientific Inc.)
- Column : Kinetex Phenomenex C18, 4.6 mm x 250 mm, 5 μm

Analytical balance: Mettler AE 50, Switzerland

Sonicator: Branson 8510, USA

Table 1. List of *C. officinale* accession 36 samples

No.	Name	No.	Name	No.	Name
1	C1	13	C13	25	Lv1
2	C2	14	C14	26	Lv2
3	C3	15	C15	27	Lv3
4	C4	16	C16	28	Lv4
5	C5	17	C17	29	Lv5
6	C6	18	C18	30	Lv6
7	C7	19	C19	31	Lv7
8	C8	20	C20	32	Lv8
9	C9	21	C21	33	Lv9
10	C10	22	C22	34	Lv10
11	C11	23	C23	35	Lv11
12	C12	24	C24	36	Li1

Table 2. Information of *C. officinale* accession samples

Name	Origin	Importer
C1	Korea	Hongildang Co., Ltd.
C2	Korea	Jecheon-hanbang Co., Ltd.
C3	Korea	Yakcho-meongga Co., Ltd.
C4	Korea	Bobe-yakcho Co., Ltd.
C5	Korea	Cheongmeong-yackcho Co., Ltd.
C6	Korea	Handsherb Co., Ltd.
C7	Korea	Herbpharm Co., Ltd.
C8	Korea (Taebaek)	Forest Medicinal Resources Research Institute
C9	Korea (Jecheon)	Forest Medicinal Resources Research Institute
C10	Korea (Bonghwa)	Forest Medicinal Resources Research Institute
C11	Korea (Yeongyang)	Forest Medicinal Resources Research Institute
C12	Korea (Yeongyang)	Forest Medicinal Resources Research Institute
C13	Korea (Yeongyang)	Forest Medicinal Resources Research Institute
C14	Korea (Yeongyang)	Sunil Co., Ltd.
C15	Korea (Bonghwa)	Dongyang-herb Co., Ltd.
C16	Korea (Gyeongju)	Gyerim Co., Ltd.
C17	Korea (Yeongju)	Megaherb Co., Ltd.
C18	Korea (Andong)	Morning pharm Co., Ltd.
C19	Korea (Jeju)	Nanum pharm Co., Ltd.
C20	Korea	Ministry of Food and Drug Safety standard
C21	China	Jayeondameun Co., Ltd.
C22	China	Nanum pharm Co., Ltd.
C23	China	Megaherb Co., Ltd.
C24	China	Dongeibogam Co., Ltd.
Lv1	Korea (Bonghwa)	Forest Medicinal Resources Research Institute
Lv2	Korea (Bonghwa)	Forest Medicinal Resources Research Institute
Lv3	Korea (Bonghwa)	Forest Medicinal Resources Research Institute

Lv4	Korea (Andong)	Forest Medicinal Resources Research Institute
Lv5	Korea (Andong)	Forest Medicinal Resources Research Institute
Lv6	Korea (Andong)	Forest Medicinal Resources Research Institute
Lv7	Korea (Yeongyang)	Forest Medicinal Resources Research Institute
Lv8	Korea (Yeongyang)	Sunil Co., Ltd.
Lv9	Korea (Jecheon)	Dongeibogam Co., Ltd.
Lv10	Korea (Bonghwa)	Jowha pharm Co., Ltd.
Lv11	Korea	Ministry of Food and Drug Safety standard
Li1	Korea	Korea Medicinal Botanical Garden

3. Preparation of samples

3.1. Preparation of samples for UPLC-MS analysis

The pulverized powder (0.3 mg) was precisely weighed and transferred to an eppendorf tube. And they were extracted by ultrasonicator with 3 mL of 70% EtOH aqueous solution of HPLC grade for 60 minutes. After the extraction, the supernatant was adjusted to a concentration of 2 mg / mL by using MeOH and water of HPLC grade and then filtered with a 0.20 μm membrane filter (Minisart RC 15, Sartorius Stedim Boitech, Germany).

3.2. Preparation of samples for HPLC-DAD analysis

The pulverized powder (0.2 mg) was precisely weighed and transferred to an eppendorf tube. And they were extracted by ultrasonicator with 2 mL of 70% EtOH aqueous solution of HPLC grade for 60 minutes. After extraction, the supernatant was filtered with a 0.20 μm membrane filter (Minisart RC 15, Sartorius Stedim Boitech, Germany).

4. Method

4.1. Method of UPLC-MS analysis

The mobile phase of the liquid chromatography was carried out at a flow rate of 0.3 mL / min using water (A) containing 0.1% formic acid and ACN (B). Analysis was carried out at a column temperature of 40 °C and a sample temperature of 20 °C. respectively. The gradient condition of the mobile phase is as follows. (0-14 min gradient 10-90% B, 14-17 min isocratic 100% B). After analysis,

10% B was flowed for 3 minutes for the analysis start state and column equilibrium state.

Mass spectrometry was performed in negative and positive mode with ESI source. High purity nitrogen gas was used as the atomizer and auxiliary gas, and argon gas was used as the collision gas. The mass spectrometry range was 100-1500 *m / z*, the capillary voltage was 3.5 kV, the sample cone voltage was 45 V, and the source and desolvation temperatures were 120 ℃ and 350 ℃, respectively. Cone gas and desolvation gas flow rates were set to 50 L / hour, 800 L / hour and 0.2 sec, respectively. The MSE mode was used for CID (collision-induced dissociation) for the metabolite identification step, the low impinging energy for obtaining precursor ions was 4V, and the ramp value of strong collision energy for MS / MS fragment information was 40 to 45 V, respectively (Zheng et al., 2009). Sodium formate was used as a calibration material to ensure accuracy, and leucine enkephalin was flowed at 5 μL / min. with lock-mass. The detected values were corrected simultaneously and independently.

Chromatograms and mass spectrometric data from each sample were used to construct an in-house library using MassLynx ™ software (version 4.1, Waters Co., Milford, MA, USA). Peaks were collected for use with MarkerLynx ™ XS (version 4.1, Waters Co., Milford, Mass., USA). Peaks in the sample were expressed as retention time, mass to charge ratio (RT-*m / z*), and ionic strength was expressed as area value. The retention time range was set to 1.50-14.00 min, the mass range was set to 100-1500 Da, and the XIC Window was set to 0.03 Da. The marker intensity threshold was set at 10. The same retention time (tolerance 0.20 min) and peak to mass ratio (tolerance 0.05 Da) between samples were collected with the same ion. The peak width at 5% height and the peak-to-peak baseline noise were calculated automatically according to the integral of the peak, and the noise elimination value was set to 500. The ion intensity value of each peak collected was normalized to the sum of the ion intensities of all the peaks in each analysis.

4.2. Method of HPLC-DAD analysis

The mobile phase of the liquid chromatography was flowed at a flow rate of 1 mL / min using water containing 0.1% formic acid (A) and ACN (B). Analysis was carried out at a column temperature of 30 °C and a sample temperature of 15 °C respectively. The gradient condition of the moving phase is as follows. 0-8 min gradient 10-25% B, 8-18 min gradient 25-40% B, gradient 18-28 min 40-75% B, 28-30 min isocratic 75% B, 30-35 min isocratic 100% B After that, 10% B was flown for 5 minutes for the analysis start condition and the column equilibrium state.

B. Result and discussion

1. Secondary metabolites analysis of *C. officinale* by UPLC-MS

1.1. Annotation of metabolites

The metabolites were annotated based on high-resolution molecular formula, retention time, and MS / MS fragmentation information obtained by UPLC-QTOF MS. Molecular formula candidates were obtained within a tolerance of 10 ppm through elemental composition and from the compound database SciFinder®, *C. officinale* was used to confirm the presence of the corresponding molecular formula. And it was confirmed by references (Hagemeier et al., 1999, Li et. al., 2003, Yi et al., 2006, Lu et al., 2005, Guy et al., 2009, Zhu et al., 2017, Mileski et al., 2017).

1.1.1. *C. officinale* MS profiling

Representative UPLC-MS chromatograms of *C. officinale* extract are shown (Figure 5, 6). A total of twenty-four compounds were identified, of which four phenylpropanoid type compounds and eighteen phthalide type compounds were identified. . It was found that there was a difference in the ratio of the components between the samples. This indicated that there was a slight difference in the amount of expression between the imported sources. The metabolites identified in the *C.officinale* extract were shown in Table 3 and 4.

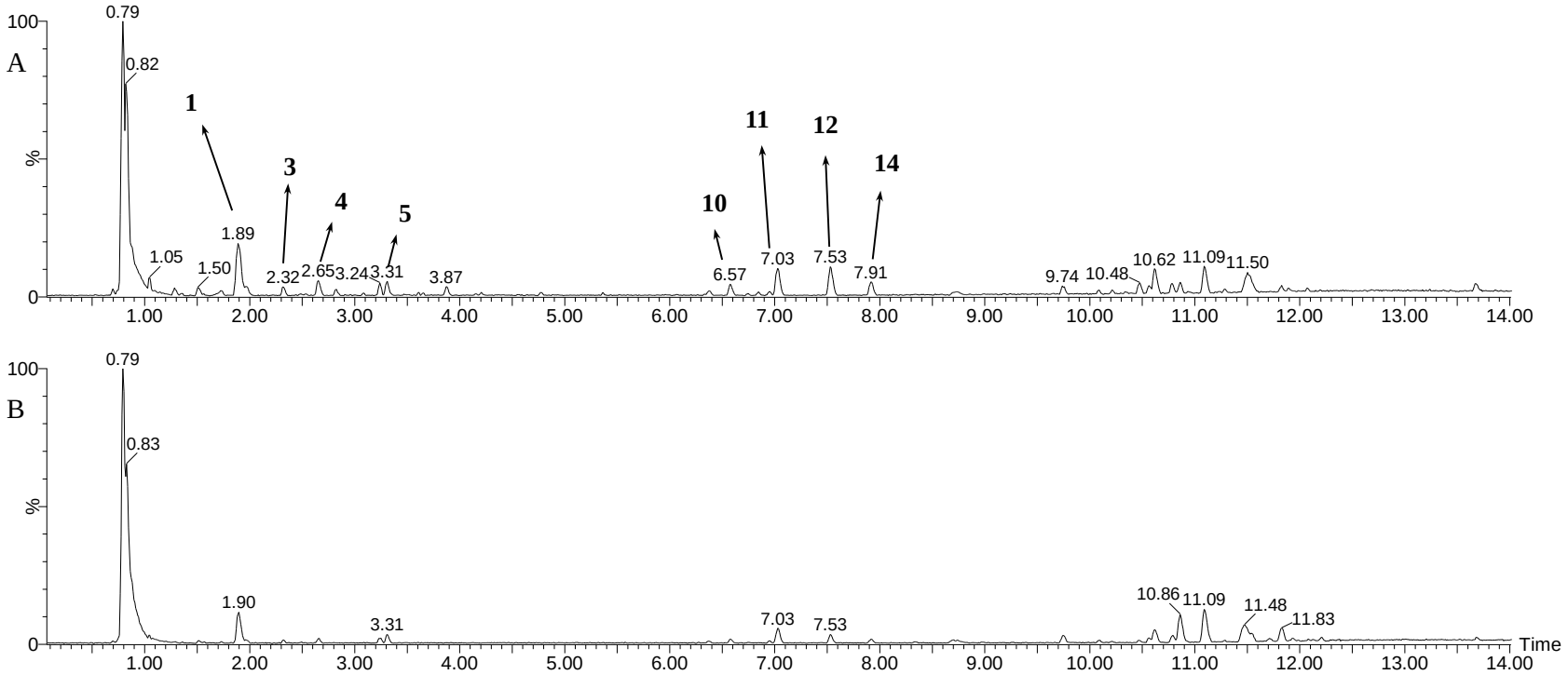


Figure 5. Representative BPI chromatograms in negative ion mode of *C. officinale* extracts. A: C3, B: C20

Table 3. Components that were identified from *C. officinale* 70% EtOH by MS negative ion mode

No.	Compound	R_t	Measured mass $[M-H]^-$	Theorical mass $[M-H]^-$	Error (ppm)	Molecular formular	MS/MS
1	chlorogenic acid	1.89	353.0875	353.0873	0.2	$C_{16}H_{17}O_9$	191
3	icariside F_2	2.32	401.1447	401.1448	-0.1	$C_{18}H_{25}O_{10}$	269, 116
4	cnidioside A	2.66	367.1035	367.1029	0.6	$C_{17}H_{19}O_9$	191
5	ferulic acid	3.31	193.0499	193.0501	-0.2	$C_{10}H_9O_4$	178, 134
10	4-hydroxy-3-butylphthalide	6.57	205.0866	205.0865	0.1	$C_{12}H_{13}O_3$	174
11	4-methoxy-3-propylphthalide	7.03	205.0862	205.0865	-0.3	$C_{12}H_{13}O_3$	161
12	senkyunolide B	7.53	203.0710	203.0708	0.2	$C_{12}H_{11}O_2$	174
14	senkyunolide C	7.92	203.0716	203.0708	0.8	$C_{12}H_{11}O_2$	174

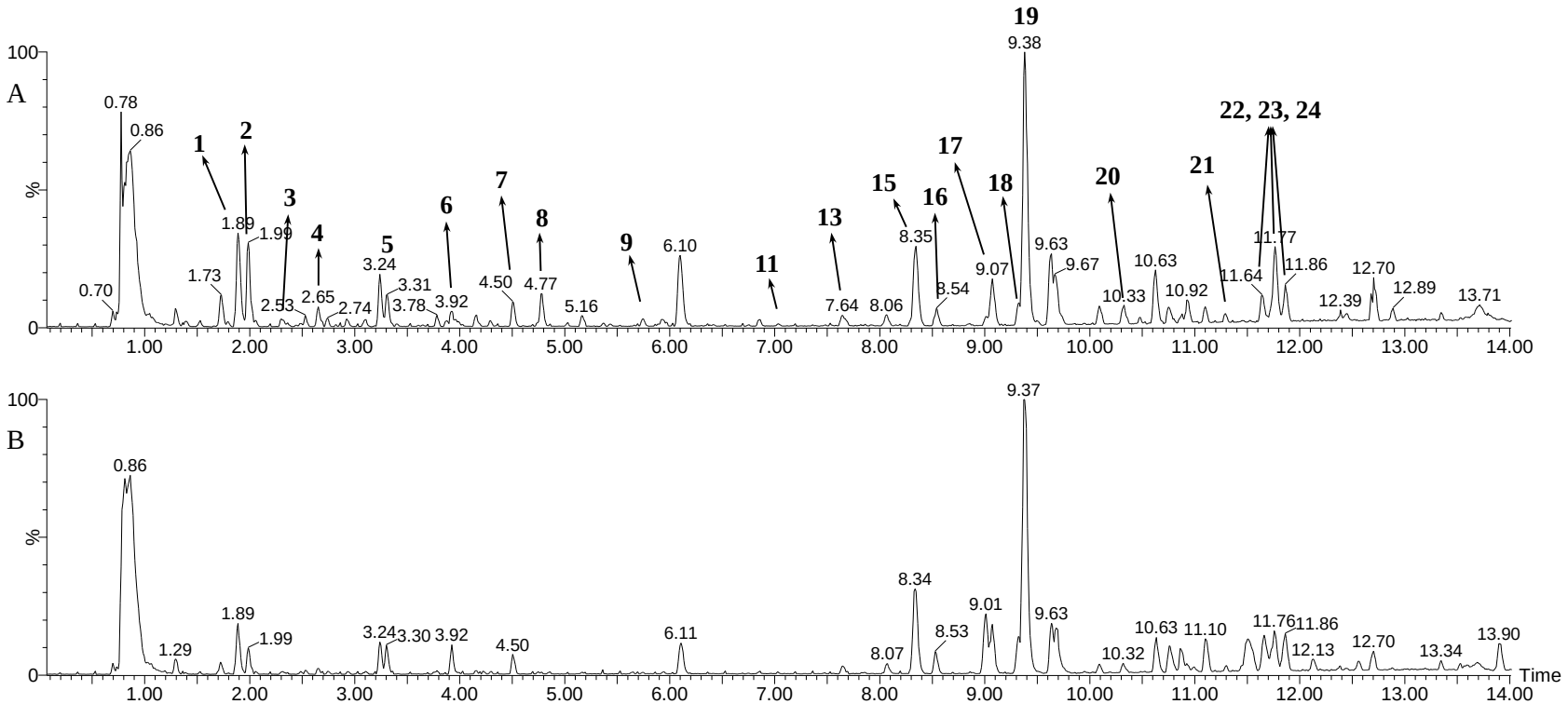


Figure 6. Representative BPI chromatograms in positive ion mode of *C. officinale* extracts. A: C3, B: C20

Table 4. Components that were identified from *C. officinale* 70% EtOH by MS positive ion mode

No.	Compound	R_t	Measured mass $[M+H]^+$	Theorical mass $[M+H]^+$	Error (ppm)	Molecular formular	MS/MS
6	senkyunlide J	3.92	249.1100	249.1103	-0.3	$C_{12}H_{18}O_4$ Na *	209, 191
7	senkyunlide I	4.50	247.0950	247.0946	0.4	$C_{12}H_{16}O_4$ Na *	207
8	senkyunlide H	4.77	247.0950	247.0946	0.4	$C_{12}H_{16}O_4$ Na *	207
9	senkyunlide F	5.75	229.0827	229.0841	-1.4	$C_{12}H_{14}O_3$ Na *	189
13	coniferyl ferulate	7.67	379.1137	379.1158	-2.1	$C_{20}H_{20}O_6$ Na *	163, 131, 103
15	senkyunlide A	8.34	193.1231	193.1229	0.2	$C_{12}H_{17}O_2$	215, 175
16	butylphthalide	8.54	191.1059	191.1072	-1.3	$C_{12}H_{15}O_2$	173, 145
17	cnidilide	9.07	195.1398	195.1385	1.3	$C_{12}H_{19}O_2$	217
18	neocnidilide	9.34	195.1382	195.1385	-0.3	$C_{12}H_{19}O_2$	217
19	*Z*-ligustilide	9.38	191.1071	191.1072	-0.1	$C_{12}H_{15}O_2$	213, 173
20	butylidenephthalide	10.32	189.0913	189.0916	-0.3	$C_{12}H_{13}O_2$	211
21	senkyunolide P	11.44	383.2219	383.2222	-0.3	$C_{24}H_{31}O_4$	405, 215, 191
22	riligustilide,	11.65	403.1887	403.1885	0.2	$C_{24}H_{28}O_4$ Na *	213, 191
23	tokinolide B,	11.77	403.1881	403.1885	-0.4	$C_{24}H_{28}O_4$ Na *	213, 191
24	levistolide A	11.86	403.1877	403.1885	-0.8	$C_{24}H_{28}O_4$ Na *	213, 191

* Sodium adduct ion $[M+Na]^+$

3.1.2. *L. chuanxiong* MS profiling

Representative UPLC-MS chromatograms of *L. chuanxiong* extract are shown (Figure 7, 8). A total of twenty four components were identified as in the chromatogram of *L. chuanxiong* extract. Of these, four phenylpropanoid type compounds and eighteen phthalide type compounds were identified. It was found that there was a difference in the ratio of the components between the samples. This indicated that there was a slight difference in the amount of expression between the imported sources. The metabolites identified in the *L. chuanxiong* extract were shown in Table 5 and 6.

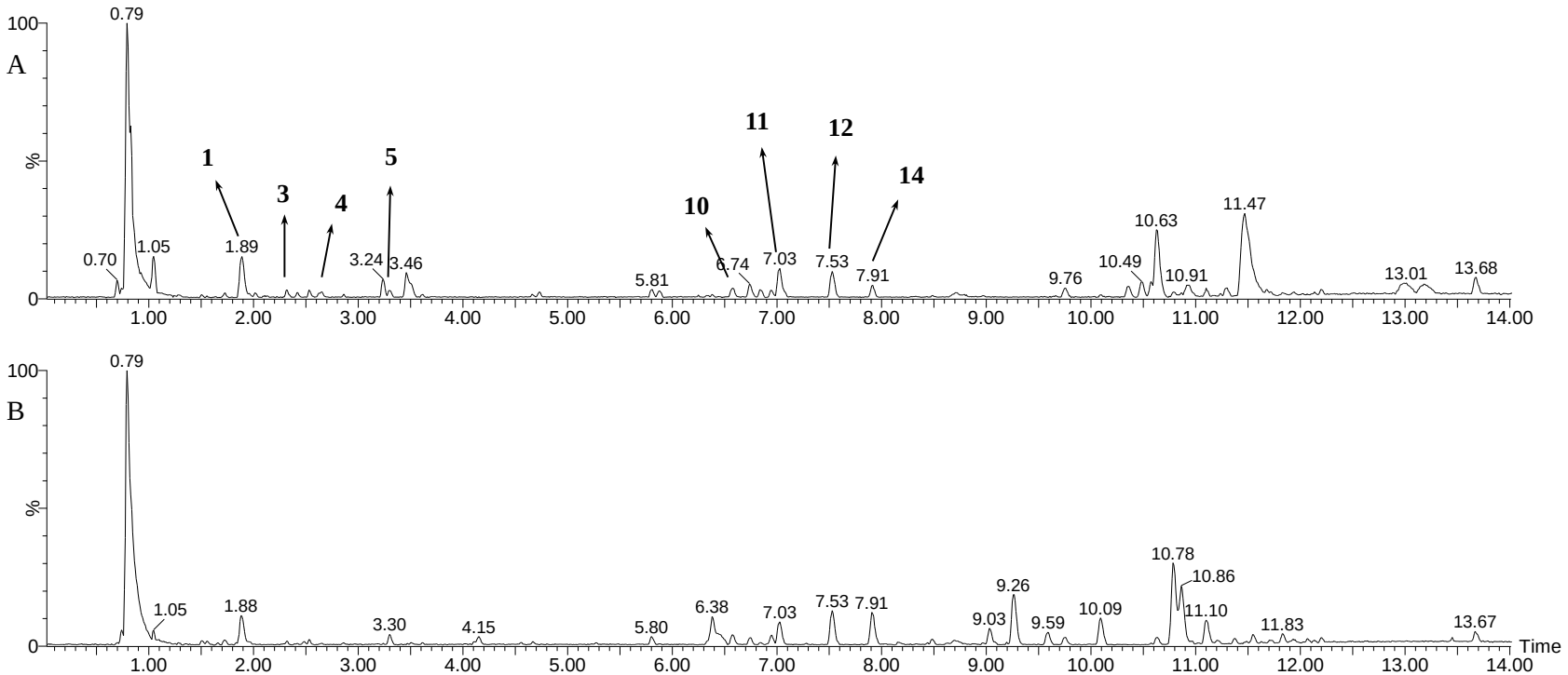


Figure 7. Representative BPI chromatograms in negative ion mode of *L. chuanxiong* extracts. A: Lv2, B: Lv10

Table 5. Components that were identified from *L. chuanxiong* 70% EtOH by MS negative ion mode

No.	Compound	R_t	Measured mass $[M-H]^-$	Theorical mass $[M-H]^-$	Error (ppm)	Molecular formular	MS/MS
1	chlorogenic acid	1.89	353.0875	353.0873	0.2	$C_{16}H_{17}O_9$	191
3	icariside F_2	2.32	401.1447	401.1448	-0.1	$C_{18}H_{25}O_{10}$	269, 116
4	cnidioside A	2.66	367.1035	367.1029	0.6	$C_{17}H_{19}O_9$	191
5	ferulic acid	3.31	193.0499	193.0501	-0.2	$C_{10}H_9O_4$	178, 134
10	4-hydroxy-3-butylphthalide	6.57	205.0866	205.0865	0.1	$C_{12}H_{13}O_3$	174
11	4-methoxy-3-propylphthalide	7.03	205.0862	205.0865	-0.3	$C_{12}H_{13}O_3$	161
12	senkyunolide B	7.53	203.0710	203.0708	0.2	$C_{12}H_{11}O_2$	174
14	senkyunolide C	7.92	203.0716	203.0708	0.8	$C_{12}H_{11}O_2$	174

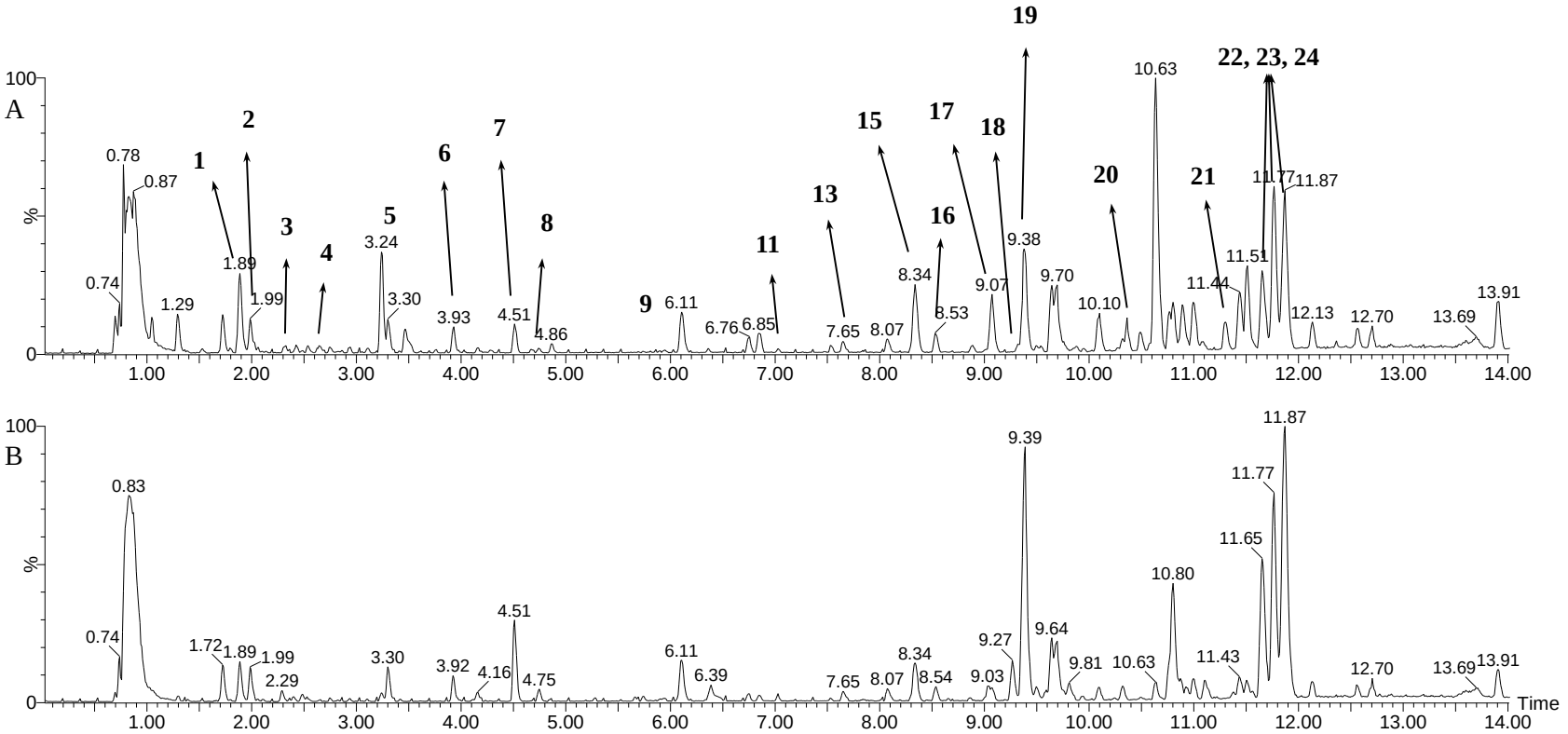


Figure 8. Representative BPI chromatograms in negative ion mode of *L. chuanxiong* extracts. A: Lv2, B: Lv10

Table 6. Components that were identified from *L. chuanxiong* 70% EtOH by MS positive ion mode

No.	Compound	R_t	Measured mass $[M+H]^+$	Theorical mass $[M+H]^+$	Error (ppm)	Molecular formular	MS/MS
6	senkyunlide J	3.92	249.1100	249.1103	-0.3	$C_{12}H_{18}O_4$ Na *	209, 191
7	senkyunlide I	4.50	247.0950	247.0946	0.4	$C_{12}H_{16}O_4$ Na *	207
8	senkyunlide H	4.77	247.0950	247.0946	0.4	$C_{12}H_{16}O_4$ Na *	207
9	senkyunlide F	5.75	229.0827	229.0841	-1.4	$C_{12}H_{14}O_3$ Na *	189
13	coniferyl ferulate	7.67	379.1137	379.1158	-2.1	$C_{20}H_{20}O_6$ Na *	163, 131, 103
15	senkyunlide A	8.34	193.1231	193.1229	0.2	$C_{12}H_{17}O_2$	215, 175
16	butylphthalide	8.54	191.1059	191.1072	-1.3	$C_{12}H_{15}O_2$	173, 145
17	cnidilide	9.07	195.1398	195.1385	1.3	$C_{12}H_{19}O_2$	217
18	neocnidilide	9.34	195.1382	195.1385	-0.3	$C_{12}H_{19}O_2$	217
19	*Z*-ligustilide	9.38	191.1071	191.1072	-0.1	$C_{12}H_{15}O_2$	213, 173
20	butylidenephthalide	10.32	189.0913	189.0916	-0.3	$C_{12}H_{13}O_2$	211
21	senkyunolide P	11.44	383.2219	383.2222	-0.3	$C_{24}H_{31}O_4$	405, 215, 191
22	riligustilide,	11.65	403.1887	403.1885	0.2	$C_{24}H_{28}O_4$ Na *	213, 191
23	tokinolide B,	11.77	403.1881	403.1885	-0.4	$C_{24}H_{28}O_4$ Na *	213, 191
24	levistolide A	11.86	403.1877	403.1885	-0.8	$C_{24}H_{28}O_4$ Na *	213, 191

* Sodium adduct ion $[M+Na]^+$

III. MS Multivariate Analysis

A. Analysis method

Multivariate analysis was performed using Simca-P + (version 13, Umetrics, umea, Sweden) program. For each variable, pareto scaling was applied to assign a small amount of ions to the ions without amplifying the noise. PCA (Principal Component Analysis) is the most representative and basic multivariate statistical analysis method for all LC-MS data. It is a useful method to identify the tendency by unsupervised method of finding the principal component by reducing dimension (Suberu et al., 2016, Worley and Powers, 2013).

In this study, major secondary metabolites from *C. officinales* accessions were identified by analysis of HRMS spectrum data were identified. As a result of comparing PCA with the data processed by MassLynx and the data of ID, only the markers could be found easily from the data of the ID only. Therefore, PCA was performed with only the identified components. After confirming whether the PCA results were clustered, the OPLS-DA (Orthogonal Partial Least Squares-Discriminant Analysis) was further added to evaluate the clear discrimination between the other groups. OPLS-DA is a supervised method which increases the influence of variables contributing to discrimination by giving information of each group as dependent variables (Kim et al., 2014). To extract the most distinct difference between the groups from the S-plot of the OPLS-DA, the cut off value was set to covariance p [1] $\geq$ | 0.1 | and p (corr) [1] $\geq$ | 0.5 | . The metabolites obtained from these were confirmed to have a VIP (Variable Importance on Projection) value of 2.0 or more for more reliable detection.

B. Result and discussion

1. Multivariate analysis of *C. officinale* and *L. chuanxiong* by MS data

C. officinale accessions The PCA (Principal Component Analysis) results for all three plants (36 samples) showed poor grouping of each plant (Figure 9). Thus, in addition to *C. officinale* and *L. chuanxiong* in the PLS-DA (Partial Least Squares Discriminate Analysis) In addition, *C. officinale* and *L. chuanxiong* showed grouping (Figure 10).

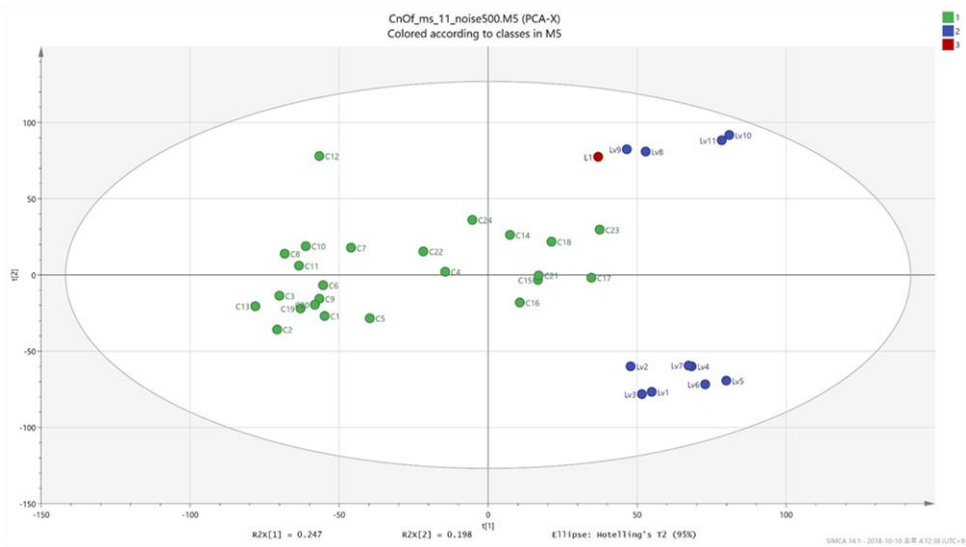


Figure 9. PCA Score plots of *C. officinale*, *L. chuanxiong* and *L. sinense* by MS data

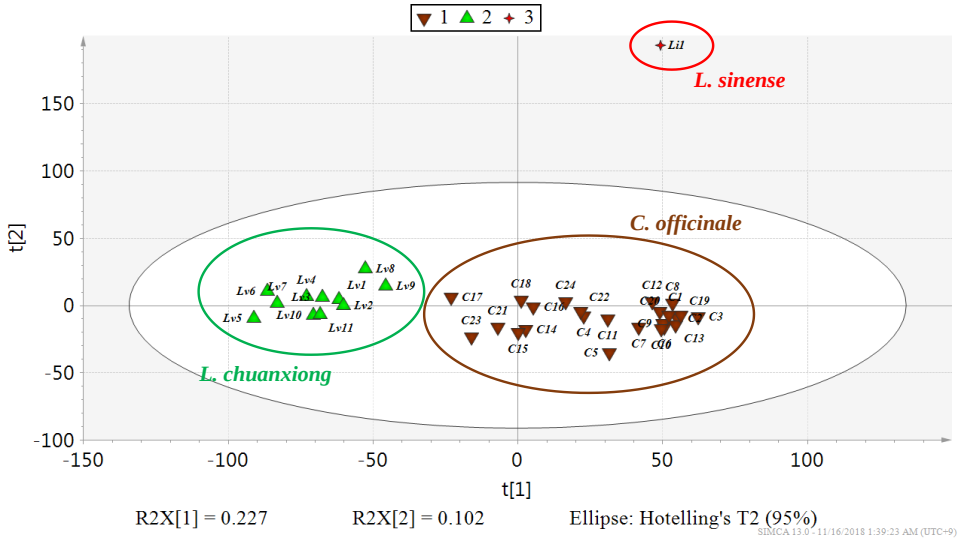
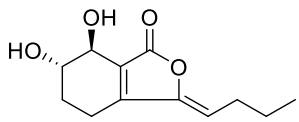


Figure 10. PLS-DA Score plots of *C. officinale*, *L. chuanxiong* and *L. sinense* by MS data

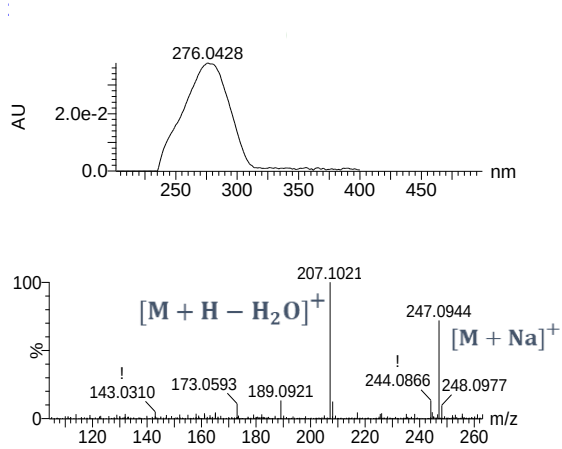
2. Comparable marker of *L. sinense* to *C. officinale* and *L. chuanxiong*

On the PLS-DA score plot, only the *L. sinense* was distantly separated from the three plants and the MS data difference with the other two plants was examined closely. *C. officinale* and *L. chuanxiong* were identical in all metabolites in the MS data, and only the metabolite content was different. However, the metabolite of *L. sinense* was different from that of *L. sinense* in MS data. In the MS chromatogram, three different metabolites were found through DAD spectrum and MS fragment (Figure 11). Among these, two compounds, senkyunolide I and senkyunolide H, were present in *C. officinale* and *L. chuanxiong* and were not detected in *L. sinense*. On the other hand, sesquiterpene bisabolangelone was detected only in L. sinense and not in the other two plants. Thus, analytical HPLC-UV was measured with these three compounds, and senkyunolide I and senkyunolide H were found to be able to be used as positive markers for *L. sinense* by *C. officinale* and *L. chuanxiong*. Bisabolangelone can be set as negative marker (Figure 12).

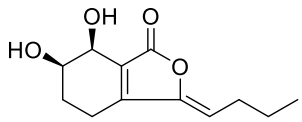
A. senkyunolide I



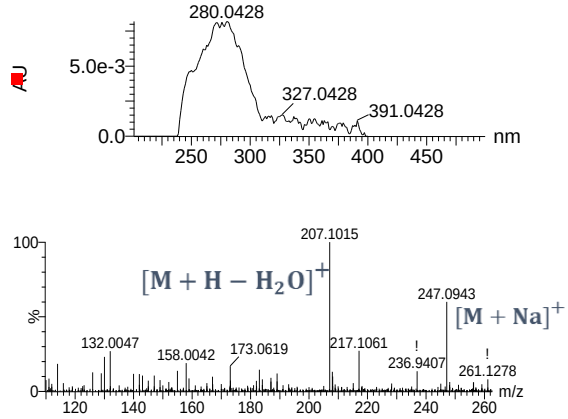
senkyunolide I



B. senkyunolide H



senkyunolide H



C. bisabolangelone

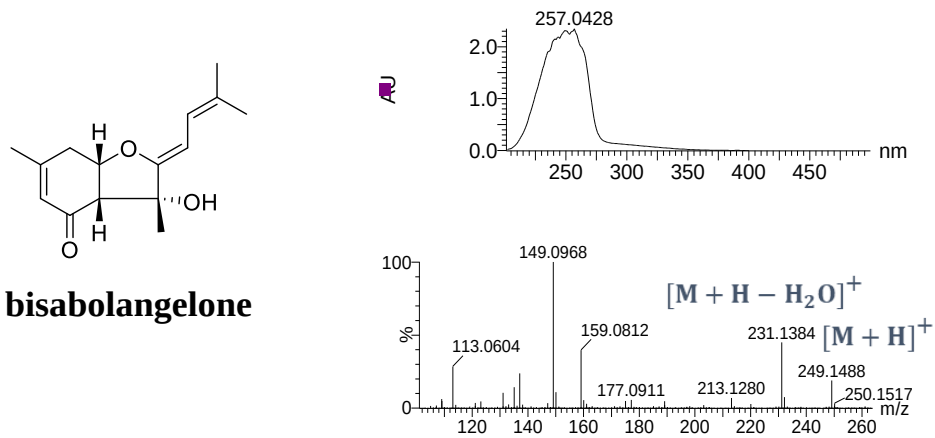


Figure 11. DAD spectrum and MS fragment of senkyunolide I (A), senkyunolide H (B) and bisabolangelone (C)

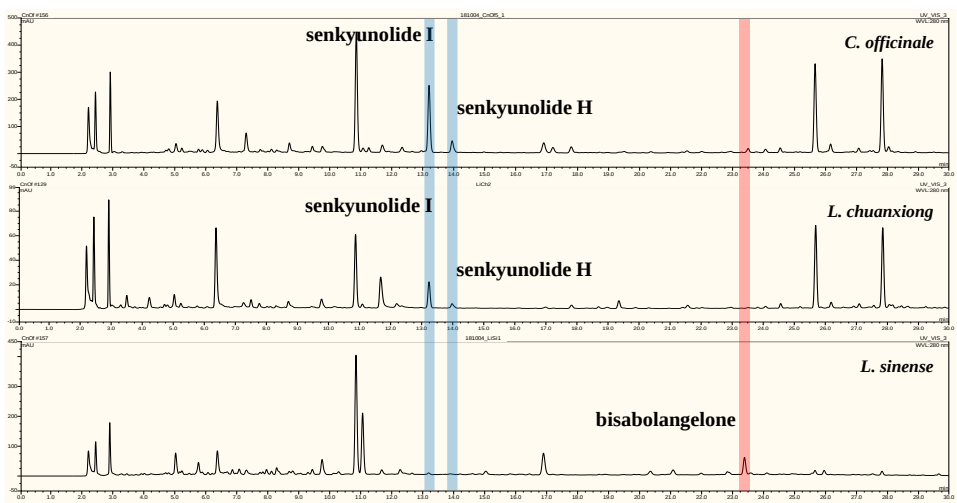


Figure 12. Comparable markers of *C. officinale* and *L. chuanxiong* to *L. sinense* by analytical HPLC-UV (280 nm)

3. Multivariate analysis of *C. officinale* and *L. chuanxiong* by MS data

C. officinale and *L. chuanxiong* were identical in all metabolites in MS data and only showed differences in content between each metabolite. Thus, we performed an additional multivariate analysis on these two plants (35 samples). First, we proceeded with PCA and saw some grouping between the two plants. In addition, they found that there were some differences in the group according to the gigantic status of the collection sources and distributors (Figure 13). A loading plot was also used to look at the metabolites contributing to the differences in each sample (Figure 14).

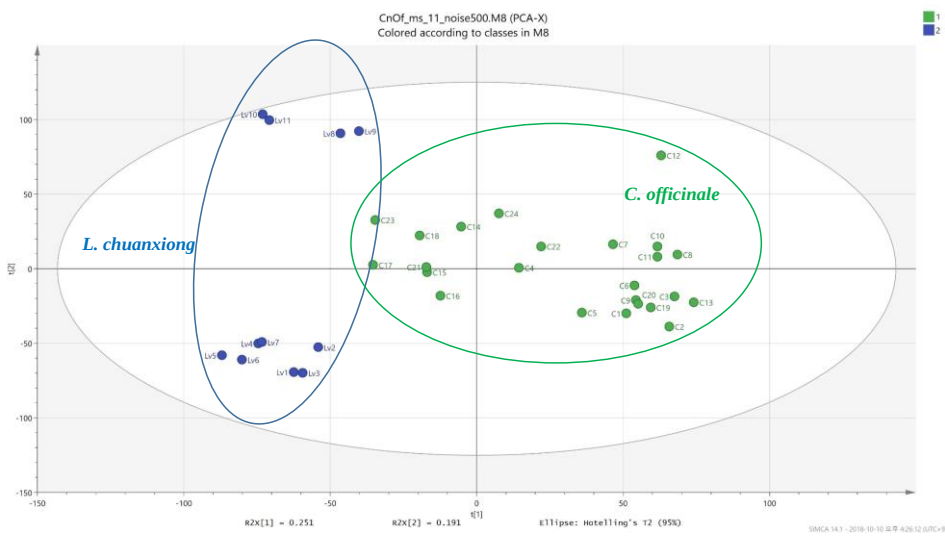


Figure 13. PCA Score plots of *C. officinale* and *L. chuanxiong* by MS data

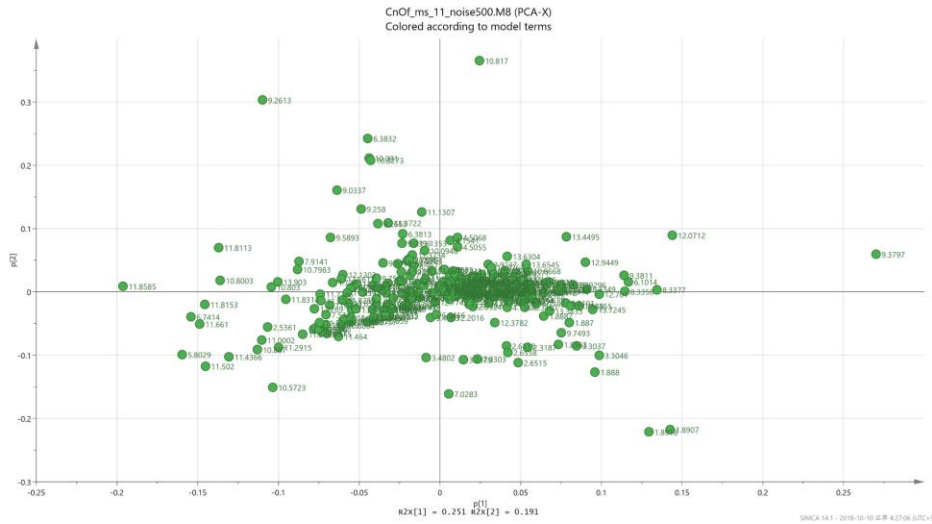


Figure 14. PCA loading plots of *C. officinale* and *L. chuanxiong* by MS data

In addition, OPLS-DA (Orthogonal Partial Least Squares-Discriminant Analysis) was performed to confirm metabolites showing the largest difference between the 2 groups through S-plot (Figure 15, 16). The markers for each group are shown in Table 8 through PCA loading plot for secondary metabolites against *C. officinale* 24 samples, *L. chuanxiong* 11 samples, and 2 groups, and S-plot of OPLS-DA. Based on the VIP score of 2.5 or higher, markers for each group consist of four ligustilide type compounds and two phenylpropanoid type compounds. Among them, ligustilide type compounds are the metabolites which are relatively more contributing to each grouping and at the same time, the less common metabolites.

As a result, it was found that Var_120 (Z-ligustilide) is the most important metabolite of two of the metabolites more contained in *C. officinale* than *L. chuanxiong*. On the other hand, Var_210, 211, and 215 (riligustilde, tokinolide B, levistolide A) were all the major metabolites of the two groupings of the

metabolites more abundant in *L. chuanxiong* than *C. officinale* as diligustilide type compounds (Figure 17).

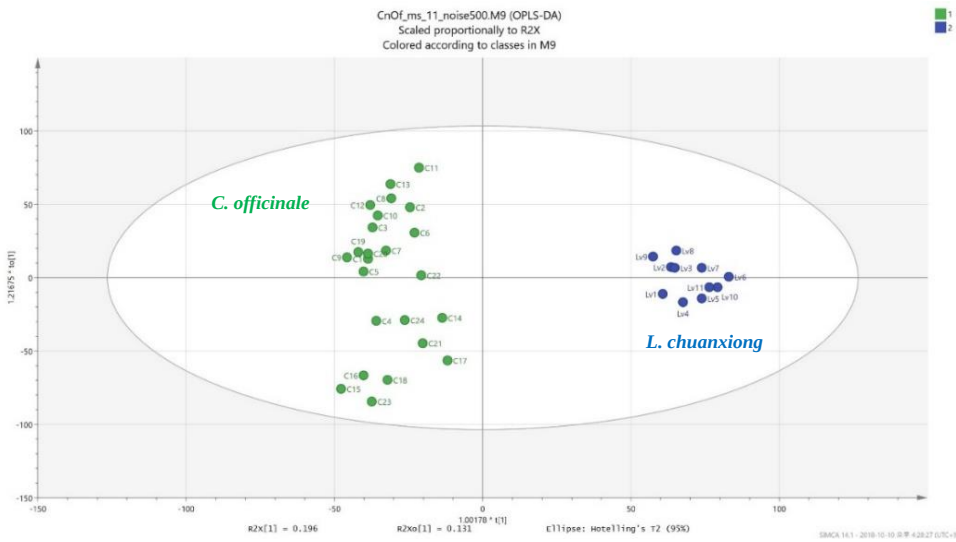


Figure 15. OPLS-DA Score plots of *C. officinale* and *L. chuanxiong* by MS data

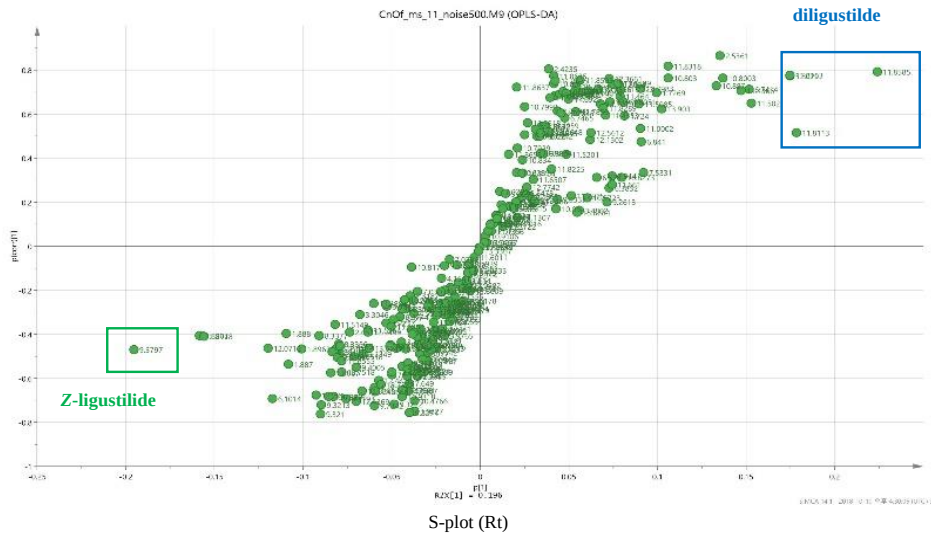
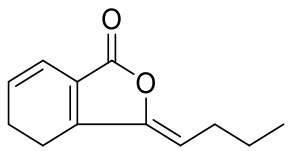


Figure 16. S-plots of *C. officinale* and *L. chuanxiong* by MS data

Table 7. Metabolic markers contributing to discrimination of *C. officinale* and *L. chuanxiong* by MS data

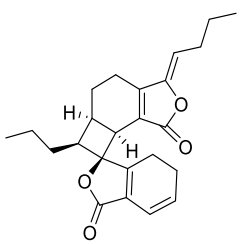
Sample	Variable no.	Proposed Identity	VIP value
C. officinale	120	*Z*-ligustilide	3.60
	7	chlorogenic acid	2.63
L. chuanxiong	215	riligustilide,	3.25
	210	tokinolide B,	3.04
	211	levistolide A	2.57
	56	coniferyl ferulate	2.56

A. ligustilide

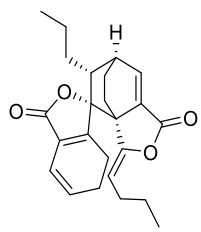


***Z*-ligustilide**

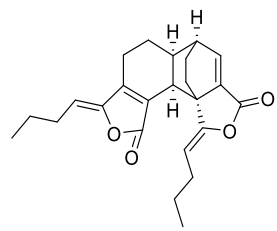
B. diligustilide type compounds



riligustilide



tokinolide B



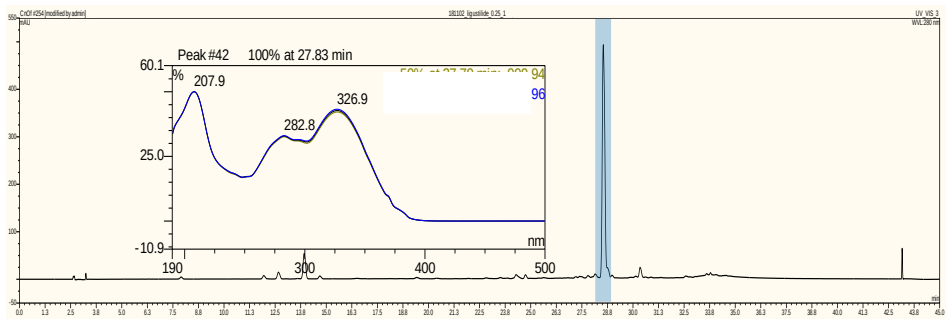
levistolide A

Figure 17. Representative metabolic markers (ligustilide type) contributing to discrimination

Three of these ligustilide type compounds, riligustilide, tokinolide B and levistolide A, are very low in *C. officinale* accessions. Therefore, UV detection does not work well. Therefore, only representative *Z*-ligustilide among the representative metabolic markers was developed by analytical HPLC-UV using the corresponding standard (Figure 18, Table 9). And, the content of *Z*-ligustilide was evaluated for *C. officinale* 24 samples and *L. chuanxiong* 11 samples by the quantitative method. Table 10 and 11 show the results of *Z*-ligustilide content of *C. officinale* accessions 35 samples. The average content of *C. officinale* was 0.096%. On the other hand, the average content of *L. chuanxiong* was 0.048%, which was

about half that of *C. officinale*. However, the *C. officinale* C10 sample was slightly lower than other *C. officinale* samples. In contrast, Lv10 and Lv11 samples, *L. chuanxiong*, showed slightly higher content than other *L. chuanxiong* samples. Samples with slightly different contents were found to have differences in imports and distributors.

A. Specificity



B. Linearity

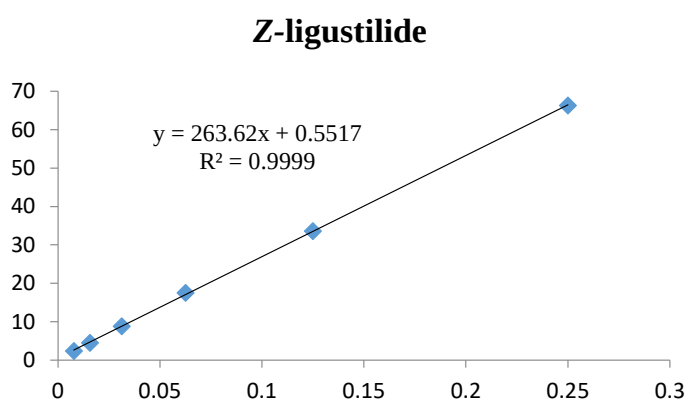


Figure 18. Quantative analysis of *Z*-ligustilide

Table 8. Analytical HPLC condition

HPLC condition			
HPLC system	Thermo Ultimate 3000 (Thermo Fisher Scientific Inc.) (PDA detector)		
Column	Kinetex C_{18} column (250 × 4.6 mm, 5 μm, Phenomenex)		
Mobile phase	Time	(A) Water (containing 0.1% formic acid, v/v)	(B) Acetonitrile (containing 0.1% formic acid, v/v)
	0.0	90.0	10.0
	8.0	75.0	25.0
	18.0	60.0	40.0
	28.0	25.0	75.0
	30.0	25.0	75.0
Flow rate	1 mL / min.		
Injection volume	10 uL		
Detection	280 nm		

Table 9. Content evaluation of *Z*-ligustilide in *C. officinale*

Origin plant	Sample	Content value (w/w %)
C. officinale	C1	0.125
	C2	0.206
	C3	0.092
	C4	0.086
	C5	0.082
	C6	0.169
	C7	0.113
	C8	0.077
	C9	0.065
	C10	0.055
	C11	0.122
	C12	0.081
	C13	0.174
	C14	0.086
	C15	0.064
	C16	0.091
	C17	0.066
	C18	0.060
	C19	0.104
	C20	0.104
	C21	0.070
	C22	0.076
	C23	0.059
	C24	0.077
	mean	0.096

Table 10. Content evaluation of Z-ligustilide in *L. chuanxiong*

Origin plant	**Sample**	**Content value (w/w %)**
L. chuanxiong	Lv1	0.042
	Lv2	0.033
	Lv3	0.045
	Lv4	0.032
	Lv5	0.041
	Lv6	0.035
	Lv7	0.038
	Lv8	0.044
	Lv9	0.061
	Lv10	0.077
	Lv11	0.078
	mean	0.048

IV. NIRS Multivariate Analysis

A. Analysis method

NIRS(Near Infrared Spectroscopy) is a spectroscopic method that uses the near-infrared region of the electromagnetic spectrum which range from 780 nm to 2500 nm (4000 cm^{-1} to 12800 cm^{-1}). It is based on molecular overtone and combination vibrations, so it can be used for identification or quantification of substances. Usually NIRS is used as typical applications including medical and physical diagnostics and research. NIRS can typically penetrate much further into a sample than mid infrared radiation. Thus, although it is not a particularly sensitive technique, it can be very useful in probing bulk material with little or no sample preparation. Since NIRS is also used for quantification, NIRS was measured for 35 samples from *C. officinale* and *L. chuanxiong*. All samples were triplicated at the time of measurement.

For sample preparation, 24 *C. officinale* and 11 *L. chuanxiong* were milled into powder with grinder for 1 minuate. The powders were passed through a 200-mesh sieve and were dried in 60 °C oven for 6 hours. NIR reflectance spectra of *C. officinale* accessions powders were obtained using an NIR system (MPA; Bruker optics, Germany) over a wavelength range 4000 cm^{-1} to 10000 cm^{-1}, using 32 scans 8 cm^{-1} resolution per spectrum. Approximately 1.0 g of the sample was filled in a glass vial. Each sample spectrum was measured three times and the final spectra were averaged. For NIRS data acquisition, spectrum preprocessing was performed by OPUS 6.0 (Bruker Optics, Ettlingen, Germany) software. In this study, several spectral preprocessing methods were used comparatively to obtain the optimum results. It includes constant offset elimination (COE), straight line subtraction (SLS), standard normal variate (SNV), multiple scattering correction (MSC), minimum maximum normalization (MMN), first derivative (1st derive),

second derivatives (2nd derive), 1st derive + SLS, 1st derive + SNV and 1st derive + MSC.

Multivariate analysis using the NIRS data was performed using Simca-P + (version 13, Umetrics, umea, Sweden) program. For each variable, pareto scaling was applied to assign a small amount of ions to the ions without amplifying the noise. Principal Component Analysis (PCA) is the most representative and basic multivariate statistical method for all of the NIRS data. It is a useful method for determining the tendency by unsupervised method of finding the principal component by reducing the dimension (Suberu et al., 2016, Worley and Powers, 2013).

Furthermore, we examined whether there is a cluster of PCA results and the samples slightly deviating from the cluster, and how much correlation there is with the content evaluation.

B. Result and discussion

1. Multivariate analysis of *C. officinale* and *L. chuanxiong* NIRS

NIRS data of 35 *C. officinale* and *L. chuanxiong* samples were converted to variable by OPUS software and the multivariate analysis was performed using SIMCA. As a result of the PCA for the data, the PCA results show that the two plants are grouping, but some samples are somewhat out of the cluster (Figure 19). As a result of performing the PLS-DA with the same data, it appears to be almost similar to the PCA result, and both plants form some grouping, but there are some samples that are somewhat deviated (Figure 20). Because of the characteristic of NIRS, the spectrum appears by molecular overtone and combination vibrations, so it is not known which variable represents the metabolite in each group. Only the

grouping using PCA and PLS-DA can be observed and compared to the quantitative result of HPLC-UV data.

By the result, especially Lv10 and Lv11 samples of *L. chuanxiong* deviate from the *L. chuanxiong* group. As shown in the results of the previous content evaluation, *C. officinale* showed a *Z-ligustilide* content of about twice that of *L. chuanxiong*. However, Lv10 and Lv11 samples, which were somewhat high in *L. chuanxiong*, showed some deviation from the group in multivariate analysis through NIRS.

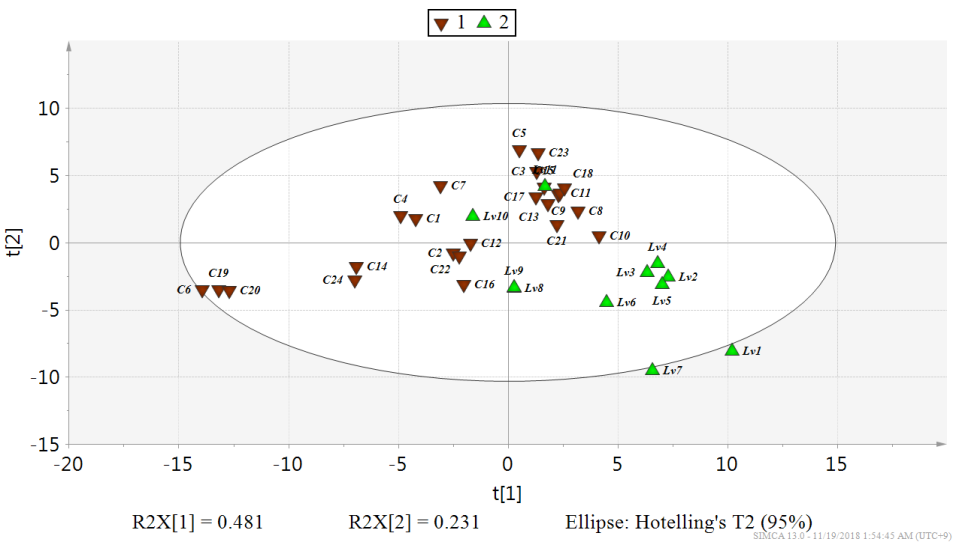


Figure 19. PCA Score plots of *C. officinale* and *L. chuanxiong* by NIRS data

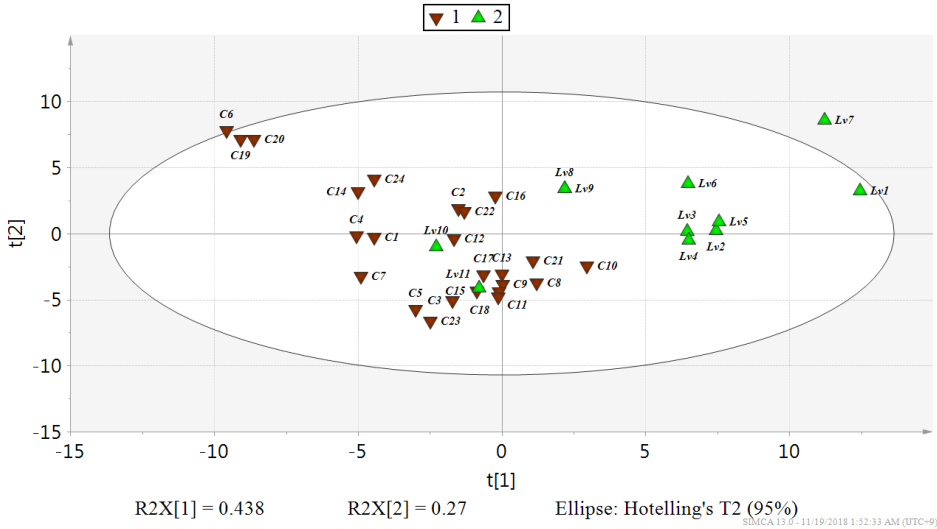


Figure 20. PLS-DA Score plots of *C. officinale* and *L. chuanxiong* by NIRS data

IV. Conclusion

1. A comparative analysis of metabolites was conducted to identify *Cnidium officinale* accessions on a physico-chemical basis. These results suggest that metabolite profiling of *C. officinale* accessions have progressed systematically.

2. UPLC-QTOF MS analysis conditions were established mainly for phthalide type compounds and each metabolite was annotated. A multivariate analysis based on secondary metabolite profiling was performed to identify patterns and trends of metabolites of *C. officinale* accessions and to identify markers that distinguish the accessions.

3. The results of the above study confirmed that it is possible to distinguish between *C. officinale* and *L. sinense* using positive and negative markers. In addition, the quantitative method of *Z*-ligustilide, which is the most comparable metabolite of *C. officinale* and *L. chuanxiong*, was developed and content evaluation was conducted to confirm the difference in content.

4. Through this study, understanding of the metabolic characteristics of *C. officinale* acccession plants was carried out. If further studies are conducted on more samples in the future, it is expected that it will be used as a basis for physicochemical differentiation of the *C. officinale* acccession plants.

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국문초록

천궁 (Cnidii Rhizoma)은 산형과 (Umbelliferae)에 속하는 식물로서 전통적으로 혈액 순환을 원활하게 하고 진통, 항염 효과 및 여성 질환에 뛰어난 효능 등의 활성이 알려져 있다. 하지만 이러한 천궁 (Cnidii Rhizoma)에 대하여 대한민국약전에서는 천궁 (*Cnidium officinale*)과 중국천궁 (*Ligusticum chuanxiong*) 두 가지 식물 모두를 기원식물로 지정하고 있다. 그에 반해 일본약국방 (JP)에서는 천궁 (*C. officinale*)만을, 중국약전 (CP)에서는 중국천궁 (*L. chuanxiong*)만을 기원식물로 설정하고 있다. 더불어 우리나라 식품공전에서도 천궁 (*C. officinale*)만을 기원식물로 지정하고 있다. 추가적으로 중국고본 (*Ligusticum sinense*) 또한 성상 및 구성 성분이 유사하여 혼용 우려가 있다. 이렇듯 나라 및 공정서 별로 천궁이라는 같은 생약명에 대한 기원식물이 상이하므로 이들 구별의 필요성이 제기된다.

그리하여 본 연구에서는 천궁 계통 식물들 사이에 대사산물의 상관성을 관찰하고자 대사체학을 기반으로 분석을 진행하였다. 천궁 (*C. officinale*), 중국천궁 (*L. chuanxiong*) 및 중국고본 (*L. sinense*) 서로의 군집 패턴을 알아보고, 군집 경향성에 영향력 있는 이차대사산물을 동정하기 위하여 UPLC-DAD-QTOF-MS 와 NIR 분광법을 사용하여 시료를 분석하였다. 분석 결과에 대하여 다변량 통계 분석 방법의 일종인 PCA 를 종 별로 시행하여 대사체가 유의미한 구별로 이어지는지를 확인하였다. 그룹 간 구별되는 경우, UPLC-MS 데이터를 기반으로 OPLS-DA 모델을 구축하고 S-plot 분석을 통해 그에 기여하는 대사체 마커를 규명하였다. 그들 중 중국고본 (*L. sinense*)는 구성 성분들 중 상이한 대사체인 senkyunolide I 및 senkyunolide H 를 MS fragment 및 DAD

spectrum 을 통하여 positive marker 로 설정할 수 있음을, 그리고 bisabolangelone 을 negative marker 로써 구별할 수 있음을 확인하였다.

천궁 (*C. officinale*)과 중국천궁 (*L. chuanxiong*)은 구성 성분이 동일하였기에, 이들의 MS data 를 활용한 PCA 및 OPLS-DA 의 S-plot 을 통해 각 군집에 가장 크게 기여하는 대사체를 확인하였다. 그리하여 phthalide 계열 성분인 *Z*-ligustilide 와 diligustilide 계열 성분들의 함량이 유의미한 구별로 이어짐을 확인하였다. 그들 중 diligustilide 계열 성분들은 해당 식물들에서 함유량이 낮고 UV detection 이 낮기 때문에 *Z*-ligustilide 에 한에서만 HPLC-DAD 를 활용하여 천궁 계통 식물들을 대상으로 ligustilide 의 정량법 개발 및 함량평가를 진행하였다. 그리고 천궁 계통 식물들을 NIR 분광법으로 측정한 data 와 함께 해당 성분들에 대한 정량 결과를 비교해보았다.

이상의 연구결과는 천궁 계통 식물들을 대상으로 혼용 우려에 대한 유의미한 구별을 대사체학 관점에서 제공하였다. 따라서 본 연구는 천궁 (*C. officinale*), 중국천궁 (*L. chuanxiong*) 및 중국고본 (*L. sinense*) 감별법의 참고가 될 수 있으며, 특정 성분의 함량이나 약리 활성에 대한 참고가 될 수 있을 것으로 사료된다.

주요어 : 천궁 (*Cnidium officinale*), 중국천궁 (*Ligusticum chuanxiong*), 중국고본 (*Ligusticum sinense*), 질량분석기 (UPLC-QTOF-MS), 근적외선분광학 (NIRS), 대사체학, 다변량 분석, PCA, PLS-DA, OPLS-DA

학 번 : 2016-21848

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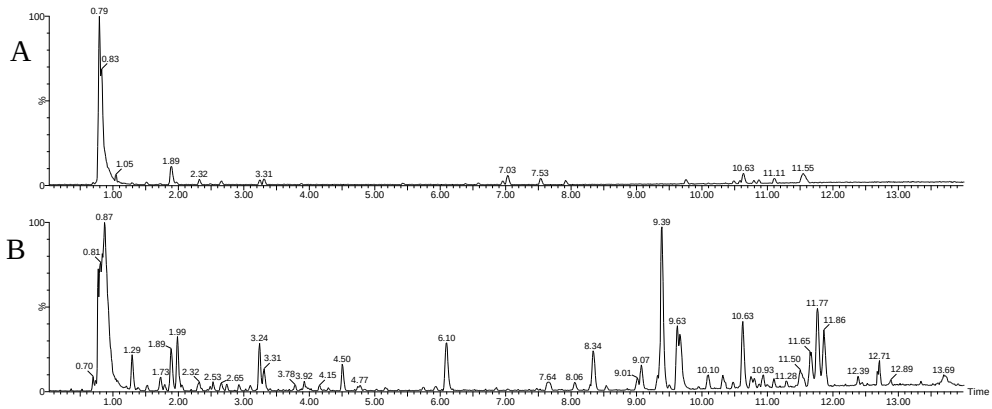


Figure S1. Chromatograms of C1 extract. A: negative ion, B: positive ion mode

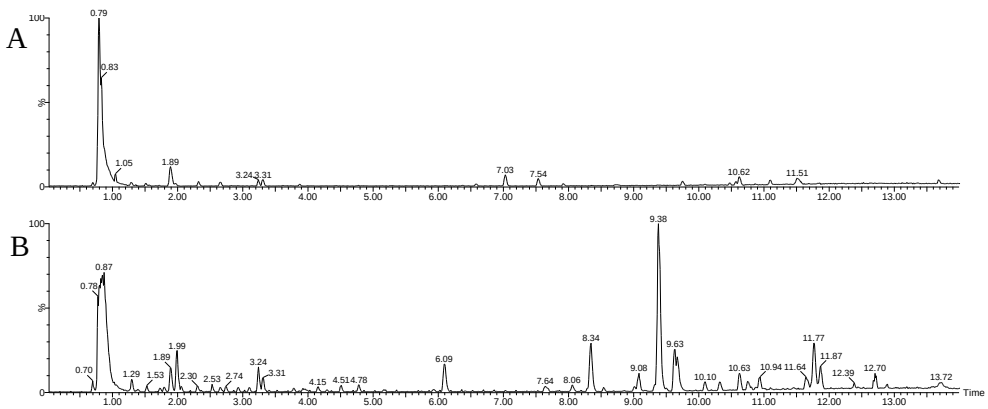
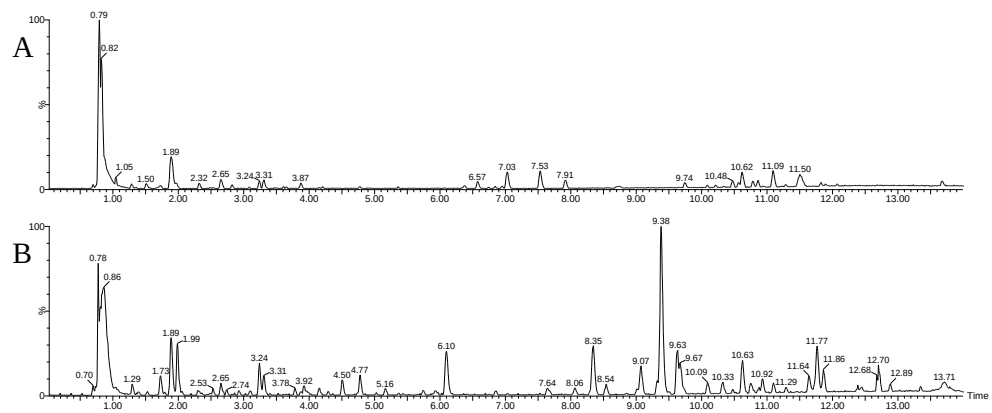


Figure S2. Chromatograms of C2 extract. A: negative ion, B: positive ion mode



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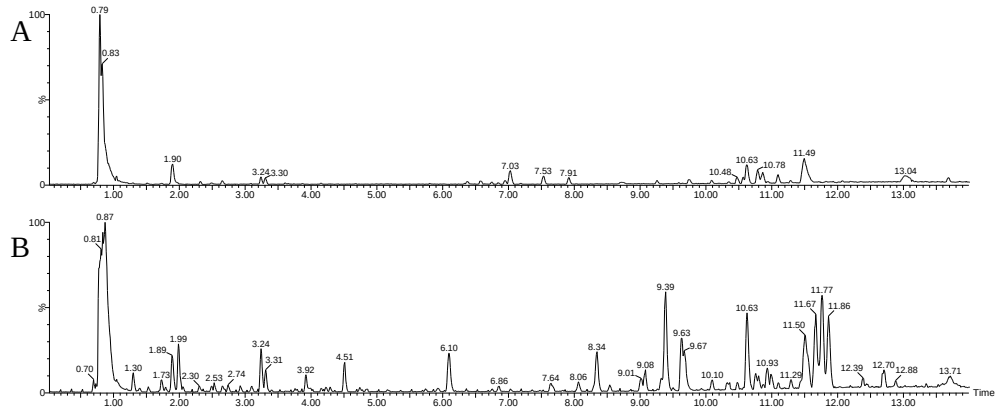

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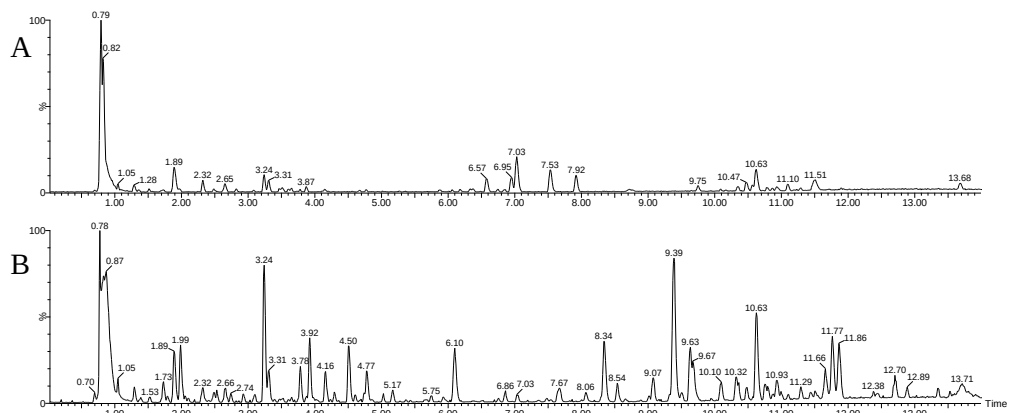

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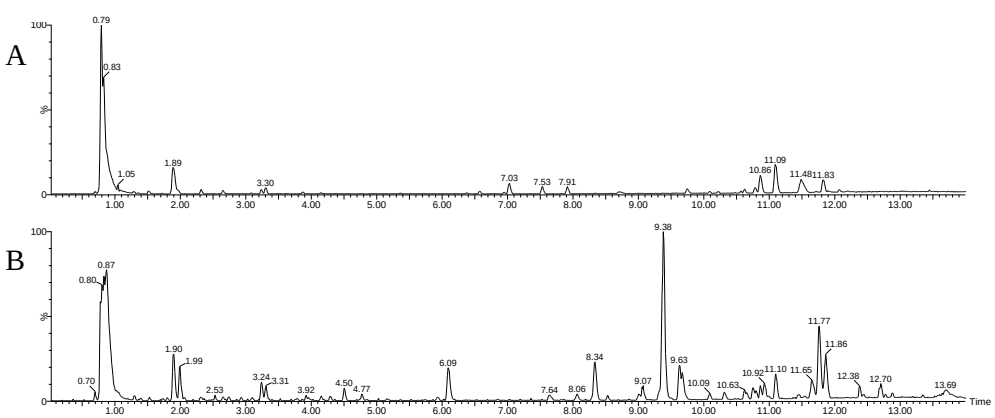

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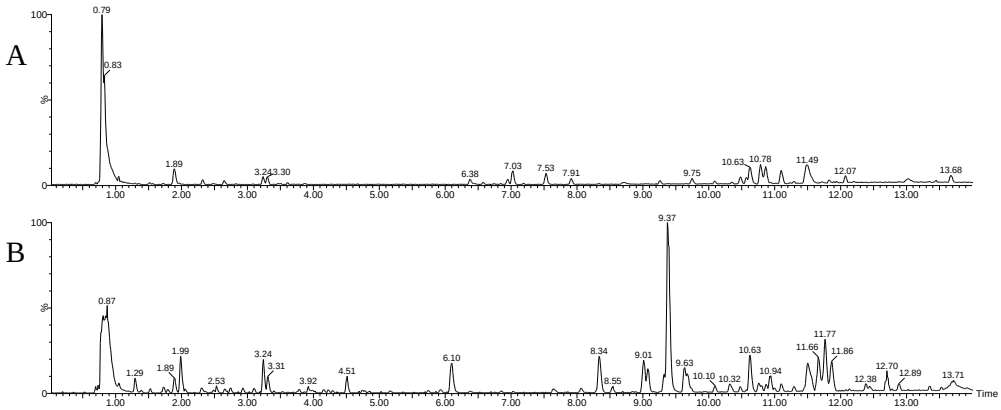

Figure S7. Chromatograms of C7 extract. A: negative ion, B: positive ion mode

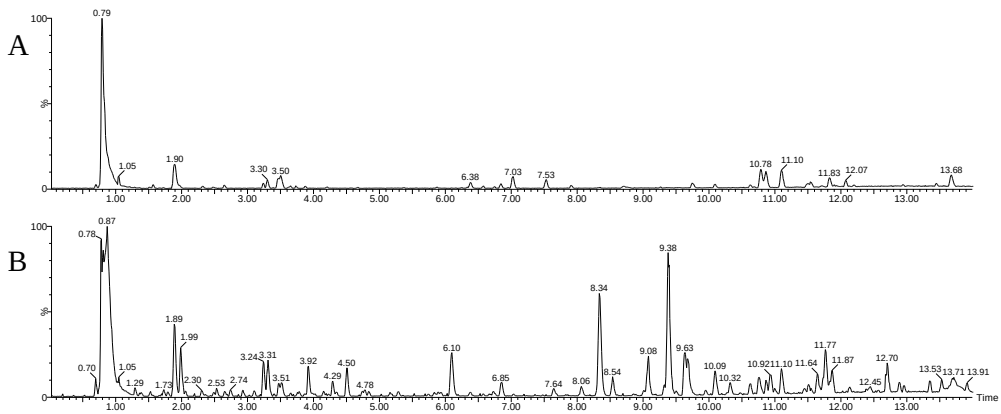

Figure S8. Chromatograms of C8 extract. A: negative ion, B: positive ion mode

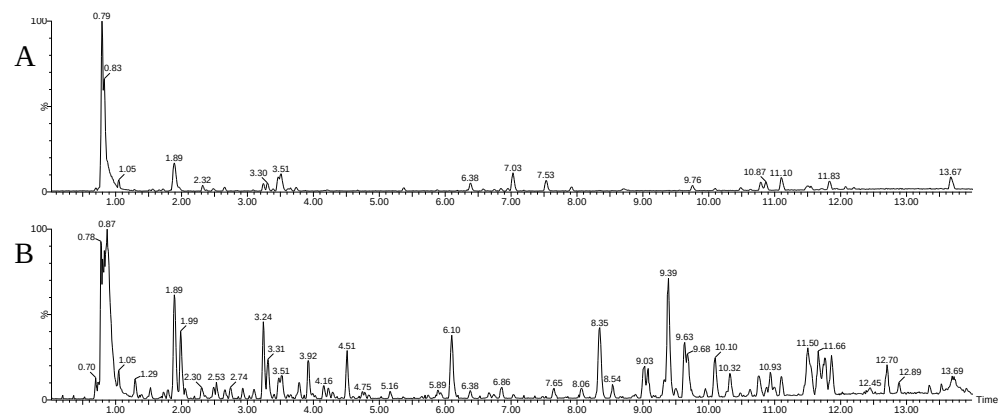

Figure S9. Chromatograms of C9 extract. A: negative ion, B: positive ion mode

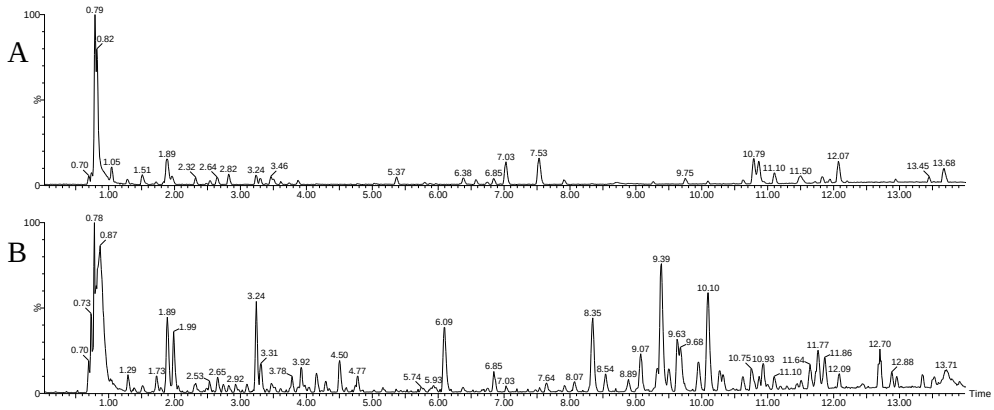

Figure S10. Chromatograms of C10 extract. A: negative ion, B: positive ion mode

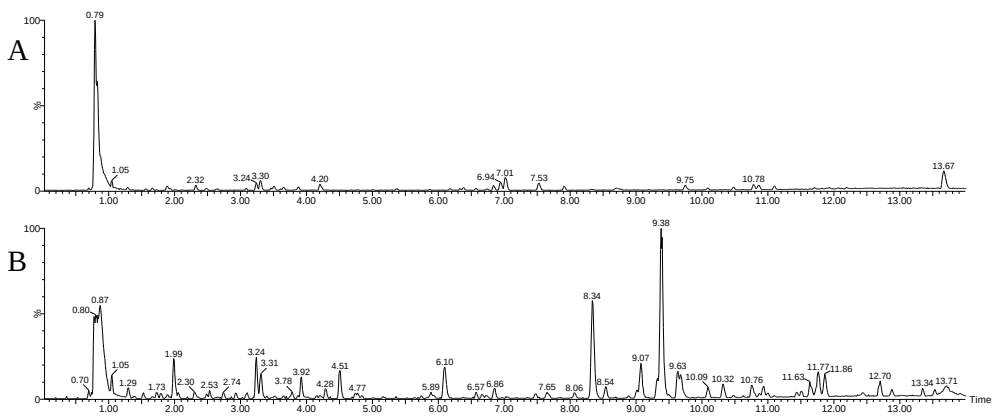

Figure S11. Chromatograms of C11 extract. A: negative ion, B: positive ion mode

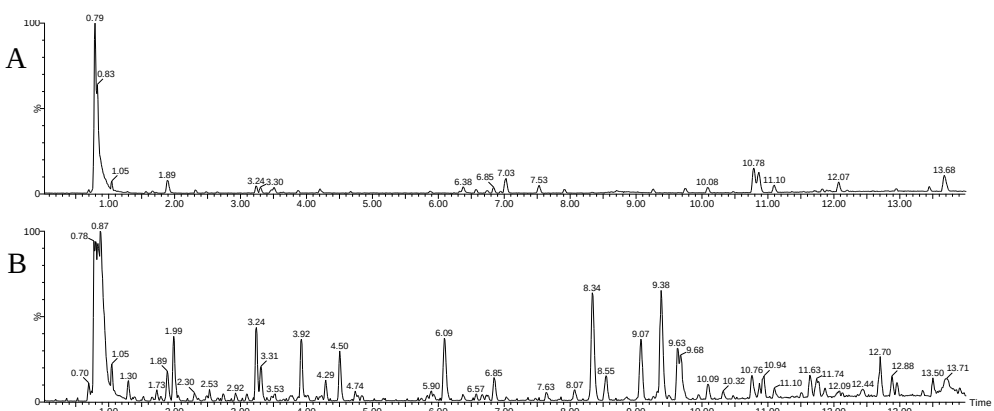

Figure S12. Chromatograms of C12 extract. A: negative ion, B: positive ion mode

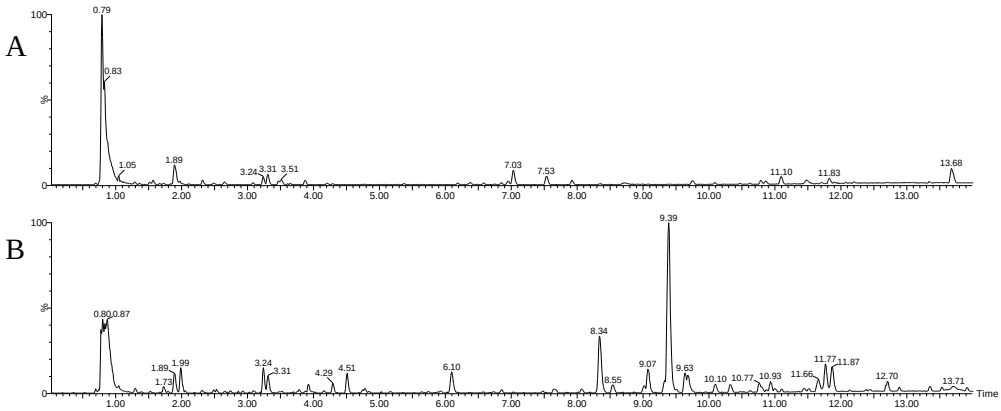

Figure S13. Chromatograms of C13 extract. A: negative ion, B: positive ion mode

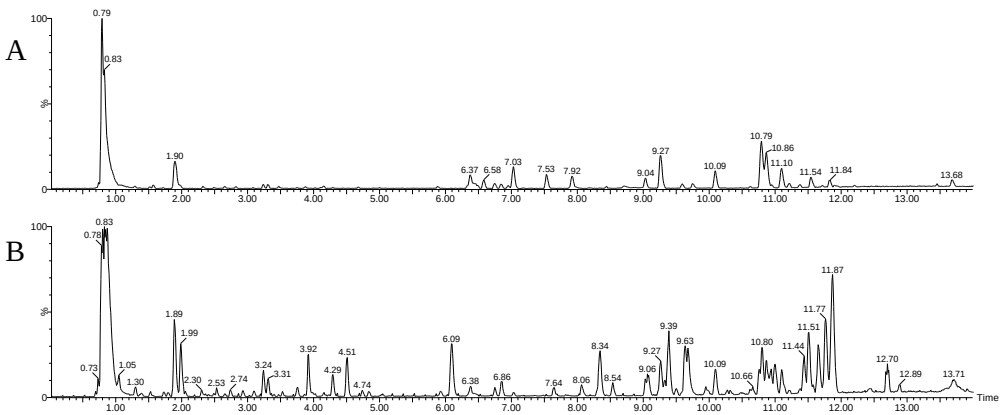

Figure S14. Chromatograms of C14 extract. A: negative ion, B: positive ion mode

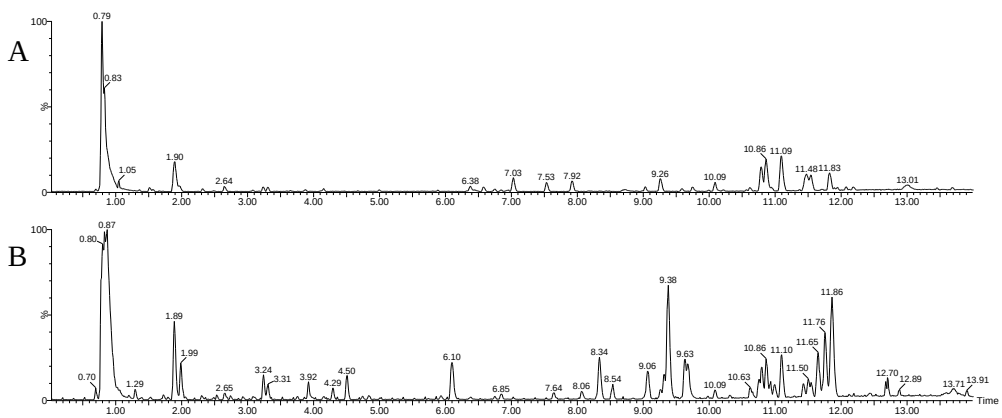

Figure S15. Chromatograms of C15 extract. A: negative ion, B: positive ion mode

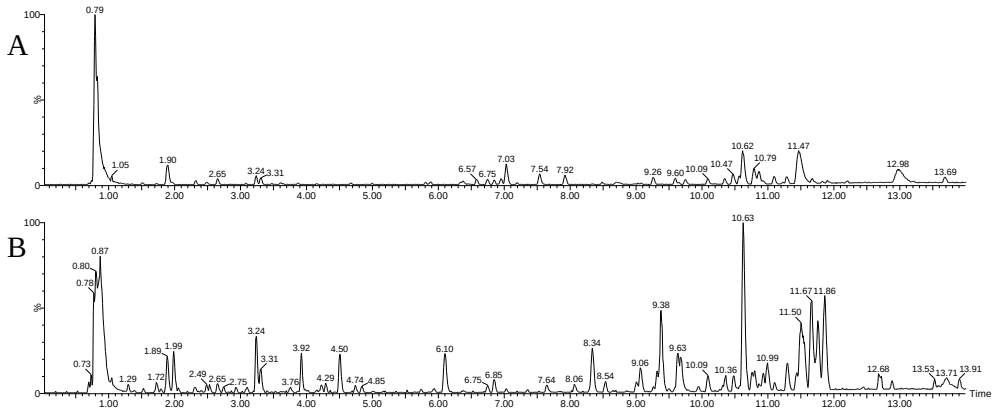

Figure S16. Chromatograms of C16 extract. A: negative ion, B: positive ion mode

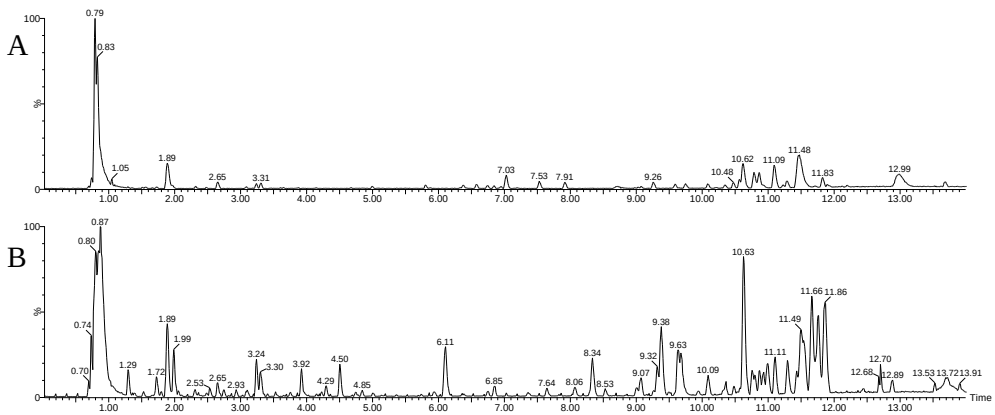

Figure S17. Chromatograms of C17 extract. A: negative ion, B: positive ion mode

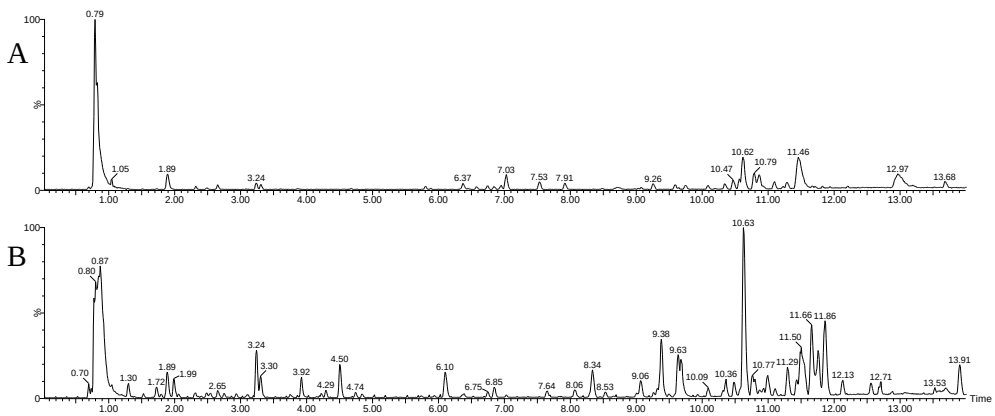

Figure S18. Chromatograms of C18 extract. A: negative ion, B: positive ion mode

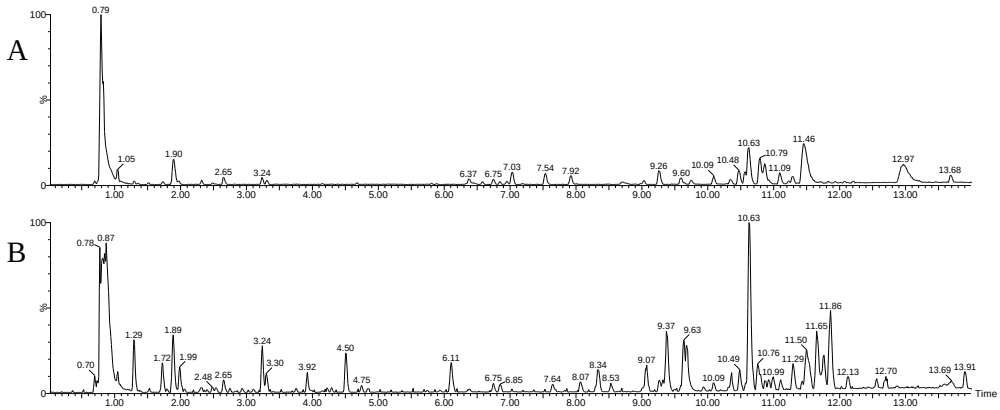

Figure S19. Chromatograms of C19 extract. A: negative ion, B: positive ion mode

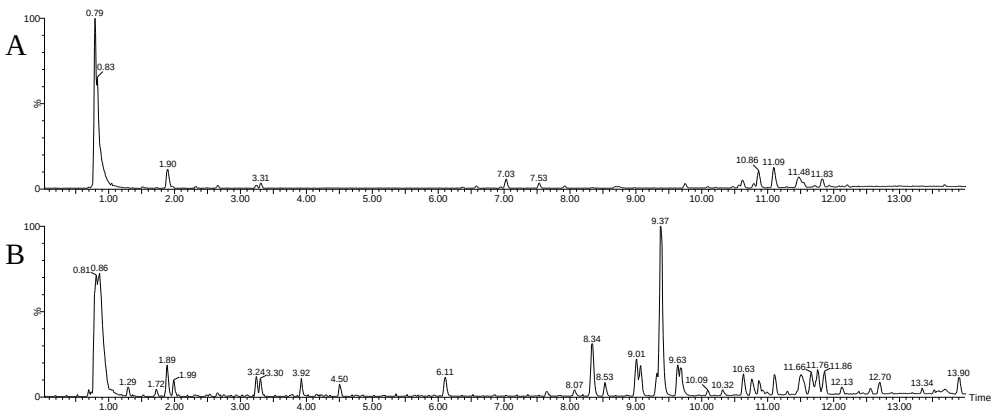

Figure S20. Chromatograms of C20 extract. A: negative ion, B: positive ion mode

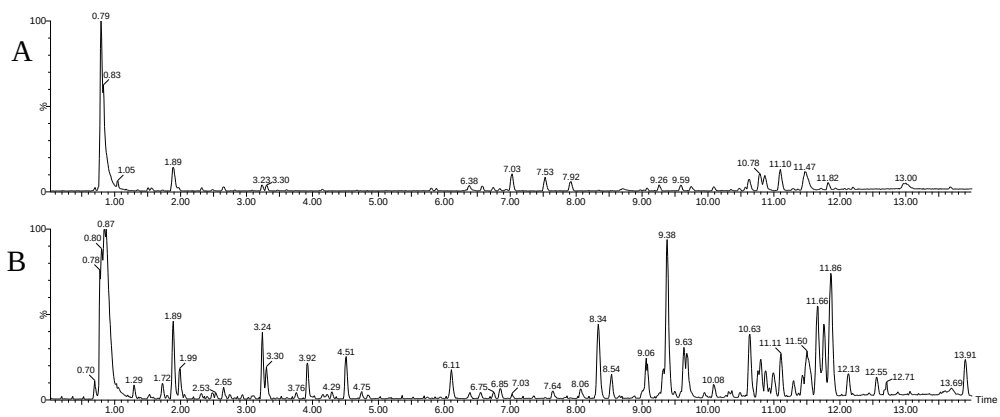

Figure S21. Chromatograms of C21 extract. A: negative ion, B: positive ion mode

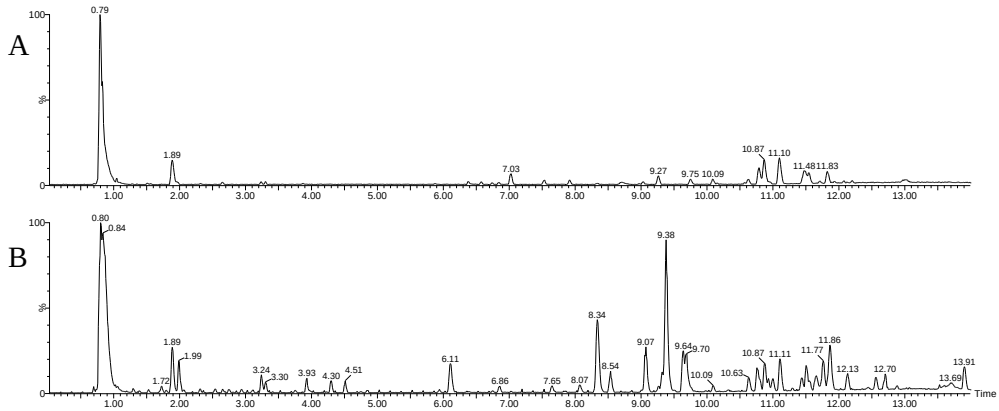

Figure S22. Chromatograms of C22 extract. A: negative ion, B: positive ion mode

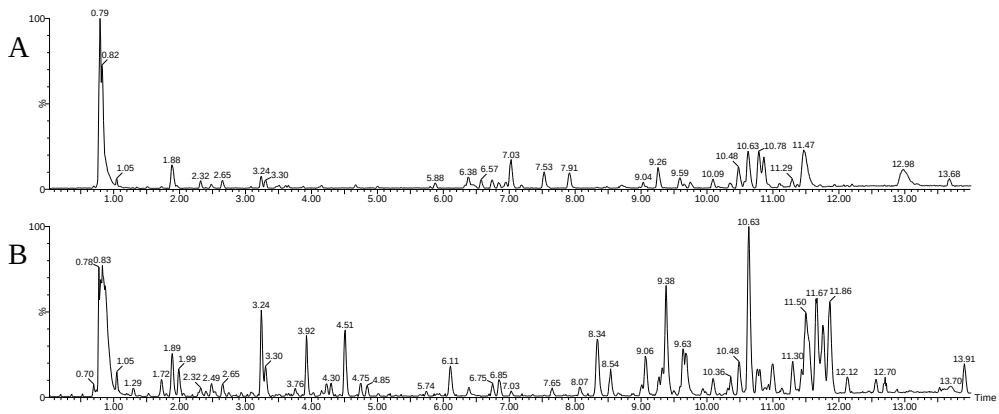

Figure S23. Chromatograms of C23 extract. A: negative ion, B: positive ion mode

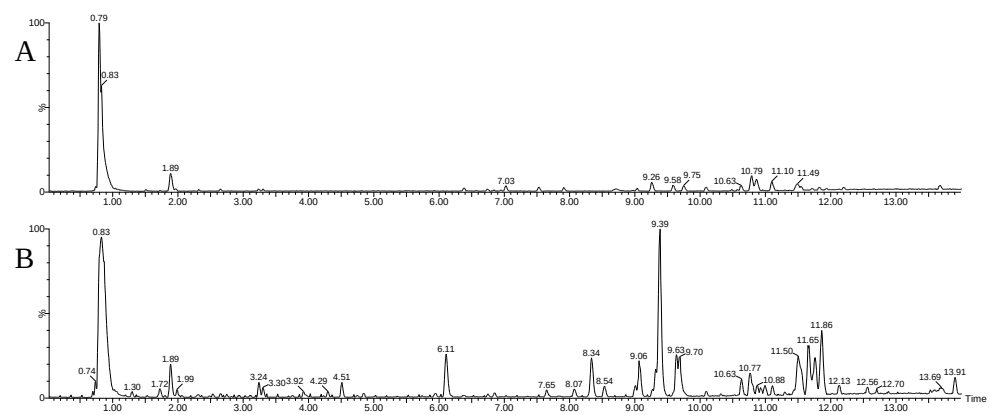

Figure S24. Chromatograms of C24 extract. A: negative ion, B: positive ion mode

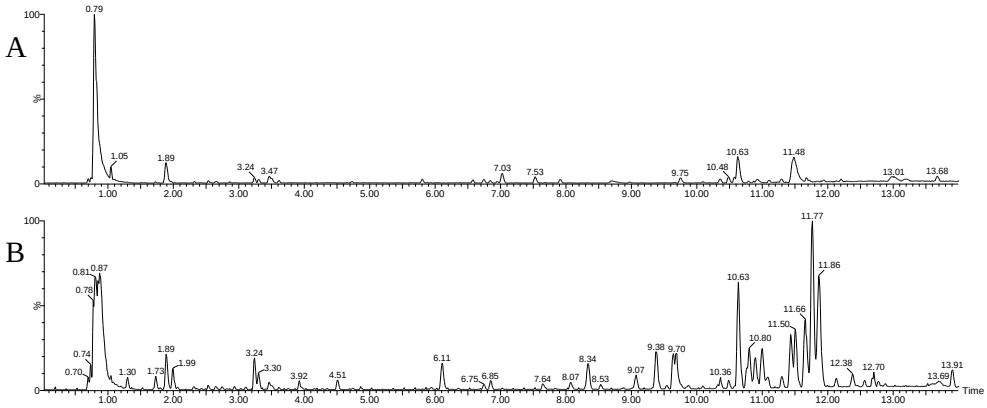

Figure S25. Chromatograms of Lv1 extract. A: negative ion, B: positive ion mode

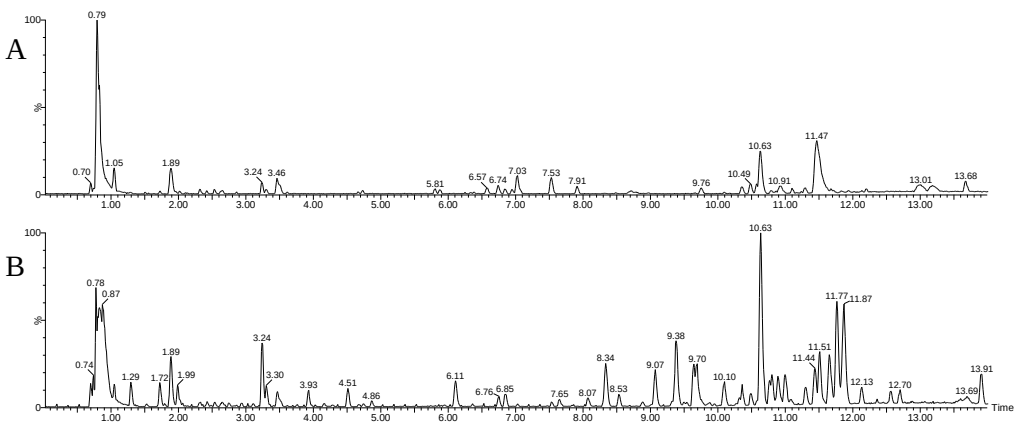

Figure S26. Chromatograms of Lv2 extract. A: negative ion, B: positive ion mode

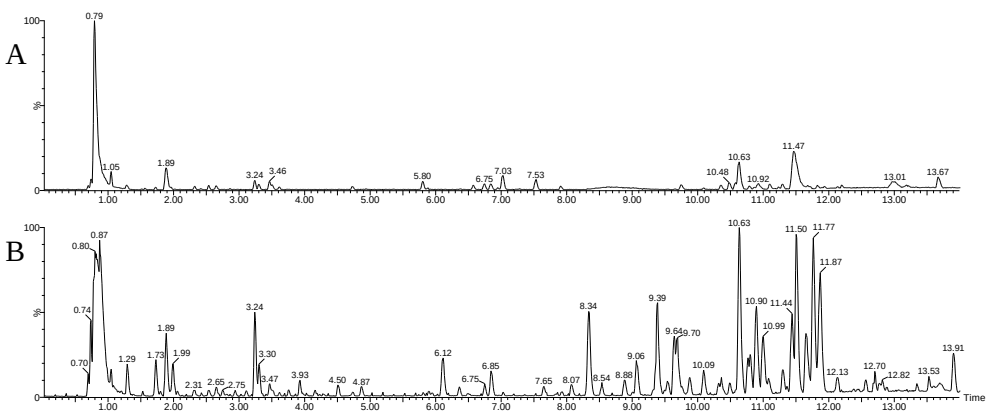

Figure S27. Chromatograms of Lv3 extract. A: negative ion, B: positive ion mode

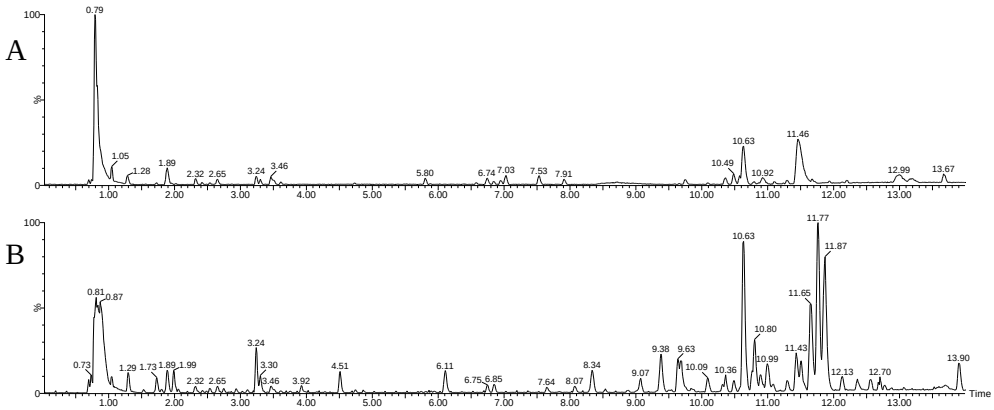

Figure S28. Chromatograms of Lv4 extract. A: negative ion, B: positive ion mode

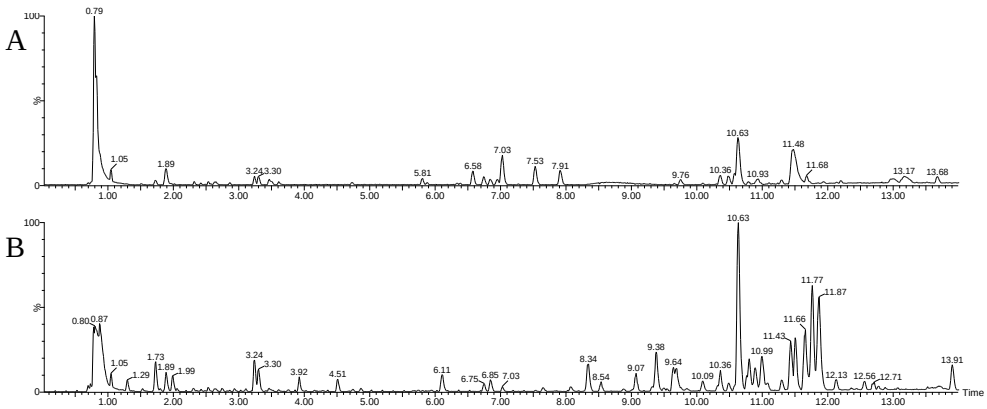

Figure S29. Chromatograms of Lv5 extract. A: negative ion, B: positive ion mode

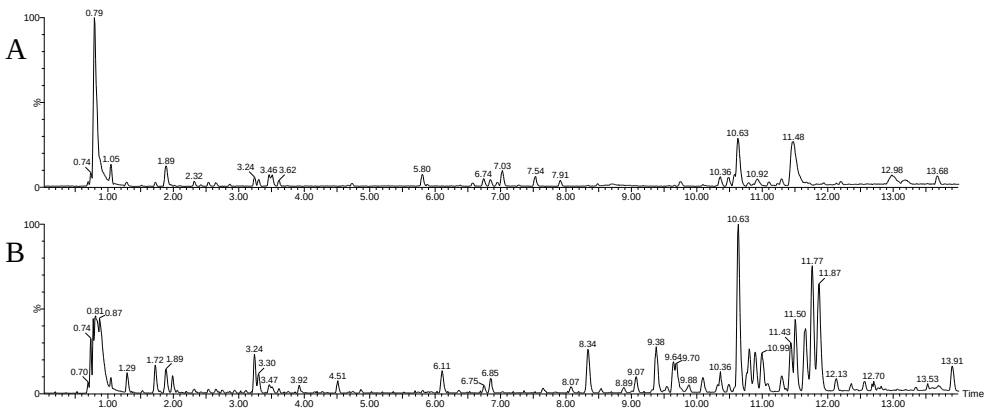

Figure S30. Chromatograms of Lv6 extract. A: negative ion, B: positive ion mode

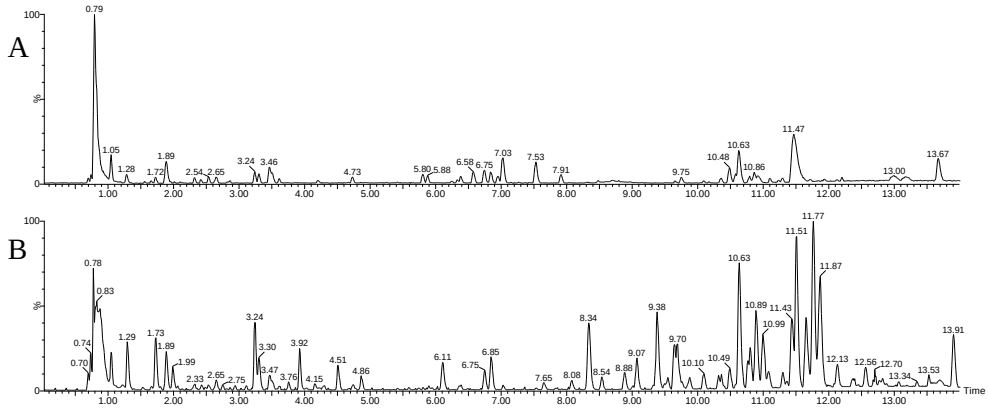

Figure S31. Chromatograms of Lv7 extract. A: negative ion, B: positive ion mode

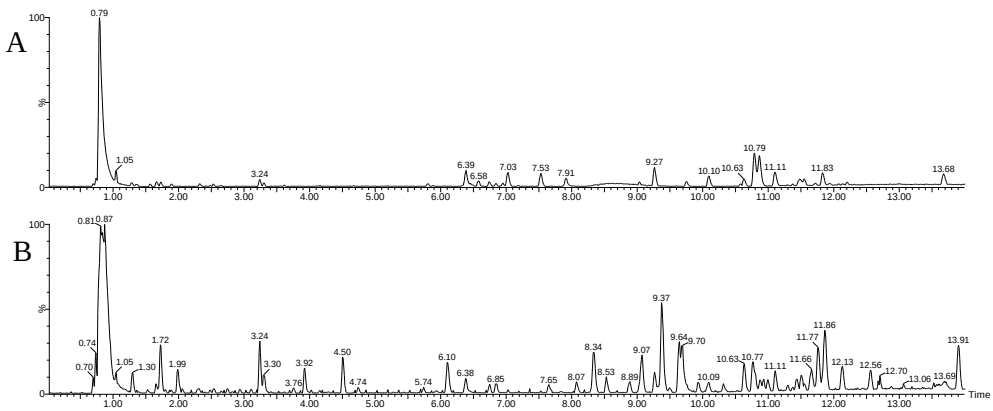

Figure S32. Chromatograms of Lv8 extract. A: negative ion, B: positive ion mode

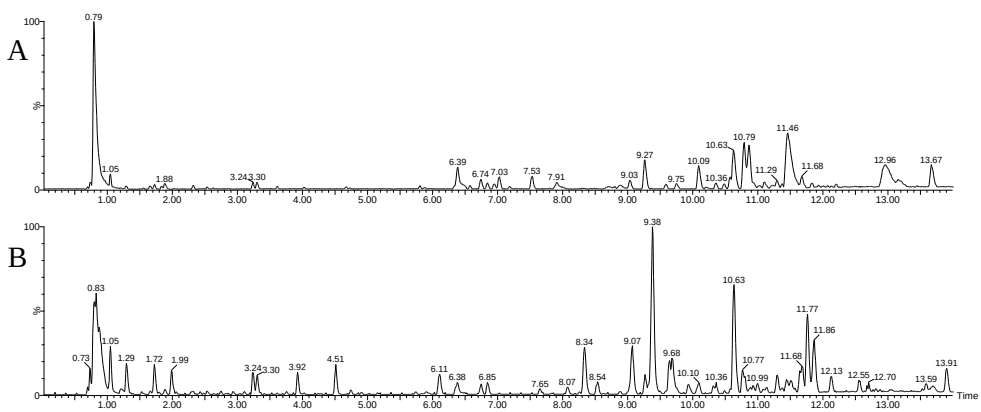

Figure S33. Chromatograms of Lv9 extract. A: negative ion, B: positive ion mode

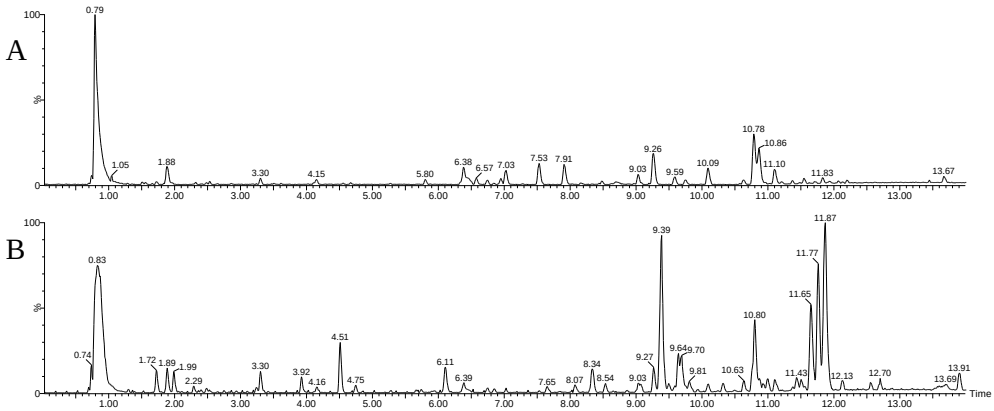

Figure S34. Chromatograms of Lv10 extract. A: negative ion, B: positive ion mode

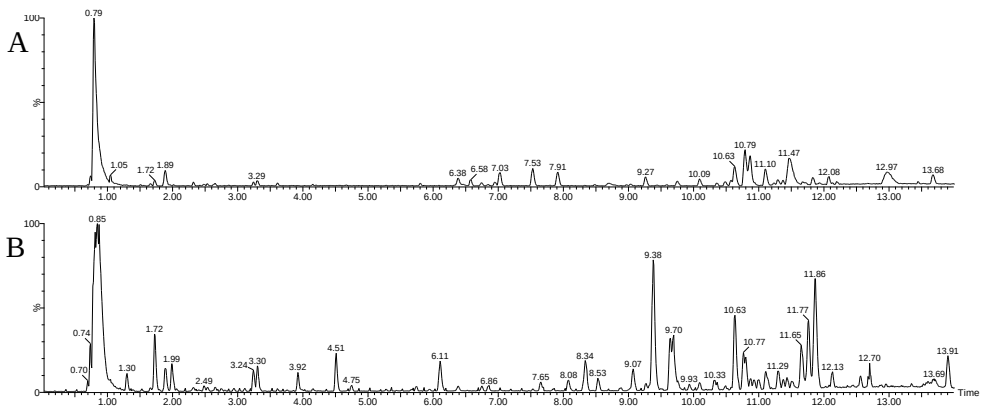

Figure S35. Chromatograms of Lv11 extract. A: negative ion, B: positive ion mode

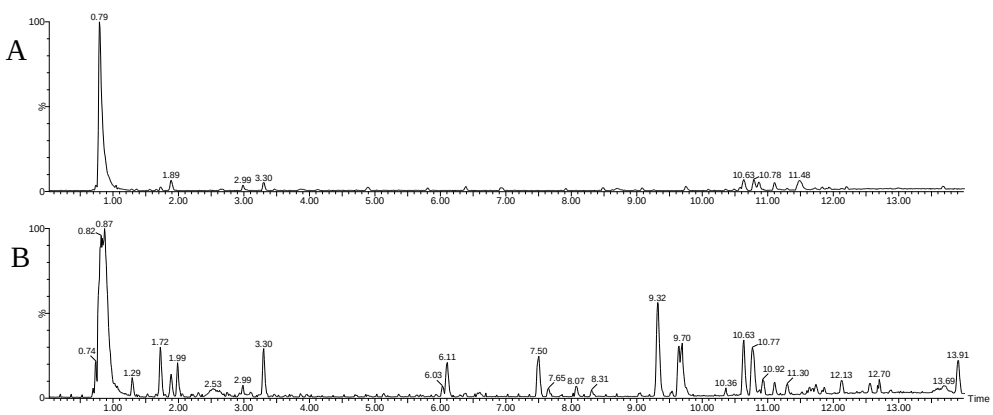

Figure S36. Chromatograms of Li1 extract. A: negative ion, B: positive ion mode