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농학박사학위논문

**Metabolomic approach for the discrimination of species,
age and processing of ginseng**

by UPLC-QTOF MS and GC-MS

**UPLC-QTOF MS 및 GC-MS를 이용한 인삼의 종,
년근 및 가공과정에 따른 대사체학 연구**

2014년 2월

서울대학교 대학원

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Thesis for the Degree of Doctor of Philosophy

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Advisor : Jeong-Han Kim

Hee Won Park

February 2014

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**Metabolomic approach for the discrimination of species,
age and processing of ginseng by
UPLC-QTOF MS and GC-MS**

Advisor: Jeong Han Kim

**A dissertation submitted in partial fulfillment
of the requirement for the degree of**

DOCTOR OF PHILOSOPHY

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**at
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ABSTRACT

An ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS) based metabolomic approach was developed in different species, steaming process and cultivation period of ginseng, respectively. And simultaneous determination method of 30 ginsenosides was established for metabolomic analysis data confirmation and getting basic information of ginseng samples using ultra performance liquid chromatography (UPLC). For the separation of 30 ginsenosides, optimization of eluent condition of UPLC and method validation was performed. The detection limits were 0.4 ~ 1.7 mg/L and calibration curves of peak area for the 30 ginsenosides were linear over three orders of magnitude with a correlation coefficients greater than 0.999. The accuracy of the method was tested by recovery measurement of the spiked samples which yielded good results of 89-118%.

Metabolomic approach was performed in order of decreasing phenotype difference of ginseng because it was hard to discriminate groups having similar phenotypes. At first, the validity of metabolomic approach method was verified by comparison to targeted analysis result of different ginseng species.

As a result, all known biomarkers, ginsenoside Rf and pseudoginsenoside F11 were identified by the proposed metabolomic method and additional potential biomarker, 20-gluco-ginsenoside Rf was extracted from the huge amounts of global analysis data.

Metabolomic analysis of *in-situ* steaming process samples, fresh ginseng, steamed ginseng and red ginseng, was performed with established method. Principal component analysis (PCA) result showed clear separation between three types of ginseng, it means that there were substantial differences in chemical compositions according to steaming process. Four potential markers, (ginsenoside 20(S)-Rg2,

20(R)-Rh1, Rh4 and arginyl-fructose-glucose), were identified. Targeted analysis of arginyl-fructose-glucose represent arginyl-fructose-glucose (AFG) generated in steaming process. There was no AFG in fresh ginseng and the average contents of AFG in steamed ginseng and red ginseng was 26.15 ± 6.99 and 65.90 ± 13.10 mg/g, respectively.

Metabolomic analysis of fresh ginseng roots by GC-MS clearly indicates that there are substantial differences in non-polar metabolites compositions according to cultivation period (4-6 years). PCA of non-polar metabolites showed clear separation between 4 and 6 years old fresh ginseng. Major constituents in non-polar fraction were polyacetylenes (30%), free fatty acids (38%), and monoacyl glycerols (17%). Short chain organic acids, terpenes, and plant steroid comprised approximately 3% of total metabolites. Correlation analysis between metabolites suggested that some fatty acids (C18:2) and monoacylglycerol are important precursors of ginseng polyacetylenes. In addition, strong positive correlation between dehydrocrepenynic acid and others polyacetylenes precursors indicates that polyacetylene biosynthesis in ginseng root may follow the previously reported pathways in other Araliaceae plants.

Finally, UPLC-QTOF MS based metabolomic approach was performed to differentiate of processed ginseng (red ginseng) with different cultivation period (4 and 6 year). Multivariate analysis, including PCA and orthogonal partial least squared discriminant analysis (OPLS-DA) of metabolites showed clear separation between 4 and 6 years old ginseng roots. Ginsenoside malonyl Rb1 was confirmed that important metabolites of this differentiation. In targeted analysis, the contents of ginsenoside malonyl Rb1 were high in all of the 6 years cultivation samples than in 4 years samples. The average contents of malonyl ginsenoside were 1.84 and 2.58 mg/g at 4 year and 6 year group respectively.

Key words: metabolomics, UPLC-QTOF MS, GC-MS, ginseng species, *in-situ* steaming process, age of ginseng.

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INTRODUCTION

Panax ginseng C. A. Meyer (*Panax ginseng*) has long been used in the eastern Asia, particularly in Korea and China as a traditional medicine in maintaining physical vitality. It belongs to the genus *Panax* in the family Araliaceae. *Panax ginseng* C. A. Meyer was once known as *Panax schinseng* Nees, and later renamed by the Russian scientist Carl Anton Meyer in 1843 (Yun TK., 2001 and Nomar EW. *et al.*, 2012). The name of ginseng is derived from a Chinese term referring to the “man-like” shape of the root. The genus name *Panax* was derived from the Greek word ‘panakos’ which means “all-healing” or “cure-all” (William AM. *et al.*, 2013). Historically, the use of ginseng started 4500 years ago, and its first record was written in the Chinese material medical book “ShennongBenCao Jin” about 2000 years ago (Baeg IH. *et al.*, 2013, Wei W. *et al.*, 2012). Thirteen species of ginseng were included under the genus name *panax* (Table 1, Yun TK., 2001, Soldati F., 2000). Nowadays, the production of ginseng is approximately 8000 tons per year with the therapeutic herbs consumed in 35 countries and a global market that has been estimated at about 2000 million dollars. Most of this production is limited to two genera of ginseng (*Panax ginseng* and *Panax quinquefolius*), and four countries: South Korea, China, Canada, and the United States are the biggest producers in the world (Baeg IH. *et al.*, 2013).

The ginseng seeds are harvested from plants of four to five years of age. To promote germination, they were stratified for a year. Planting is performed usually in autumn and after one year growth they are transplanted to another field. Cultivation is done around four to six years roots in South Korea and China, and usually three to four years roots and some of six years roots in Canada and US (Soldati F., 2000 and Baeg IH. *et al.*, 2013).

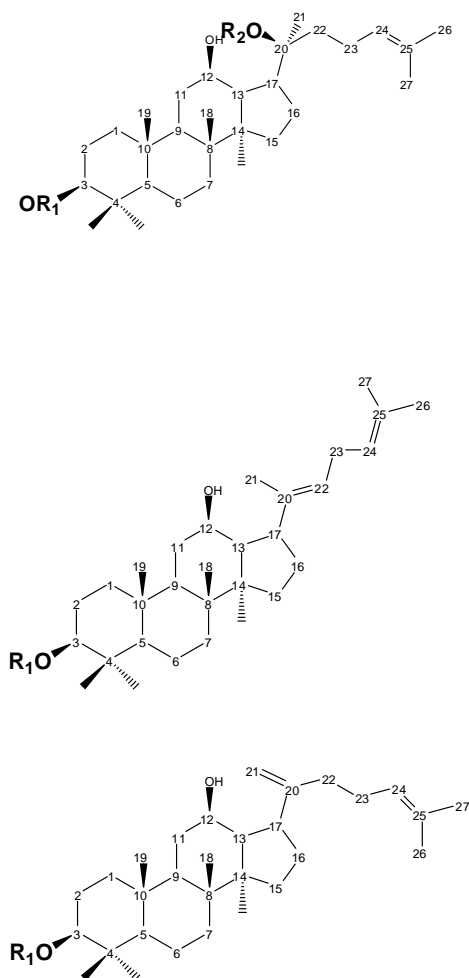
Table.1 Species under the genus name *panax*

Species	Common names	Native to
<i>Panax ginseng</i> C. A. Meyer	Asian ginseng, Chinese ginseng, Korean ginseng, Jen-shen	Eastern China and Korea
<i>Panax japonicas</i> C. A. Meyer	Japanese ginseng	Japan
<i>Panax notoginseng</i> (Burkill) F. H. Chen	Notoginseng, sanchi ginseng	South west China
<i>Panax omeiensis</i> J. Wen		
<i>Panax pseudoginseng</i> Wallich	Tienchi ginseng	China
<i>Panax quinquefolius</i> L.	American ginseng,	Eastern US and Canada
<i>Panax sinensis</i> J. Wen		
<i>Panax stipuleanatus</i> H. T. Tsai & K. M. Feng		
<i>Panax trifolius</i> L.	Dwarf ginseng, ground nut	Eastern US and Canada
<i>Panax wangianus</i> Sun		
<i>Panax zingiberensis</i> C. Y. Wu & K. M. Feng		
<i>Panax vietnamensis</i> Ha et Grushv.	Vietnamese ginseng	Vietnam
<i>Panax major</i> Ting		

Scientific research on the components of ginseng was started from separation of saponin fraction, panaquilon, by Garriques in 1854. Despite this starting, the components of ginseng were not studied again until Shibata *et al.* established the chemical structures of main prosapogenins, 20S-protopanaxatriol, 20S-protopanaxadiol in 1963 (Kim DH., 2012). Up to now more than 150 naturally occurring ginsenosides have been isolated from roots, leaves, stem, fruits and flower heads of various *Panax* genera. Especially, *Panax ginseng* C. A. Meyer, including fresh, dried, and processed ginseng, has 49 ginsenosides in roots and rhizomes, 19 ginsenosides in leaves and stems and 42 ginsenosides in fruits and flower buds respectively (Christensen LP., 2008).

The ginsenosides can be classified into two groups by the skeleton of their aglycones, namely dammarane-, divided into protopanaxadiol type (Fig.1) and protopanaxatriol type (Fig.2), and oleanane- type (Fig.3).

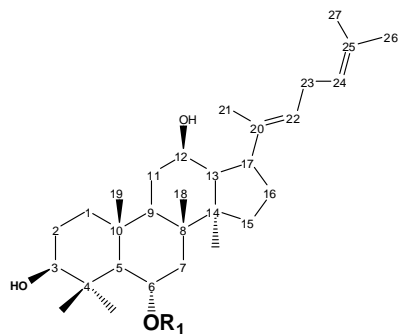
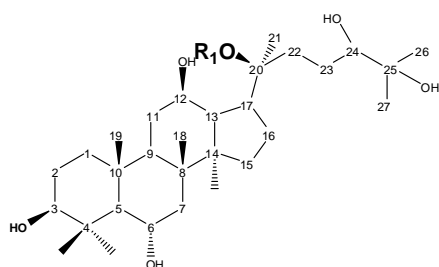
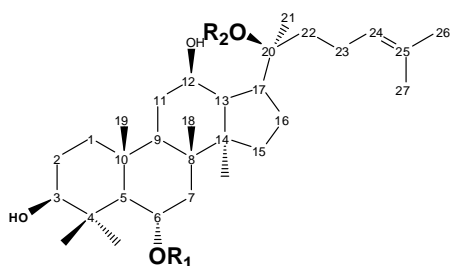
Ginseng have several classes of constituents, including ginsenosides, essential oil-containing polyacetylenes and sesquiterpenes, polysaccharides, peptidoglycans, nitrogen-containing compounds and various ubiquitous compounds such as fatty acids, carbohydrates and phenolic compounds (Nadezhda A. *et al.*, 2008). *Panax ginseng* has positive effects on the endocrine, cardiovascular, immune, and central nervous systems, prevention of fatigue, oxidative damage, mutagenicity and cancer (Soldati F., 2000 and Christensen LP., 2008). Ginsenosides are triterpenes considered to be the main bioactive constituents of ginseng and show various pharmacological effects such as anti-carcinogenic, immunostimulatory, antiatherosclerotic, antihypertensive and antidiabetic effects as well as effects on the CNS and stress (Christensen LP., 2008).



Ginsenoside	R1	R2
Ginsenoside Rb1	Glc ² -Glc	Glc ⁶ -Glc
Ginsenoside Rc	Glc ² -Glc	Glc ⁶ -Ara(f)
Ginsenoside Ra1	Glc ² -Glc	Glc ⁶ -Ara(p) ⁴ -Xyl
Ginsenoside Rb2	Glc ² -Glc	Glc ⁶ -Ara(p)
Ginsenoside Rb3	Glc ² -Glc	Glc ⁶ -Xyl
Ginsenoside Rd	Glc ² -Glc	Glc
Ginsenoside F2	Glc	Glc
Ginsenoside Rg3(20s)	Glc ² -Glc	H
Ginsenoside Rg3(20r)	Glc ² -Glc	H
Compound Y	H	Glc ⁶ -Xyl
Compound K	H	Glc
Ginsenoside Rh2(20s)	Glc	H
Ginsenoside Rh2(20r)	Glc	H
Ginsenoside	R1	
Ginsenoside Rg5	Glc ² -Glc	
Ginsenoside Rh3	Glc	

Ginsenoside	R1
Ginsenoside Rk1	Glc ² -Glc
Ginsenoside Rk2	Glc

Fig.1 Structures of protopanaxadiol type aglycone moieties and selected structure of ginsenosides. Glc, β -D-glucose; Rha, α -L-rhamnose; Ara(p), α -L-arabinose(pyranose); Ara(f), α -L-arabinose(furanose); Xyl, β -D-xylose

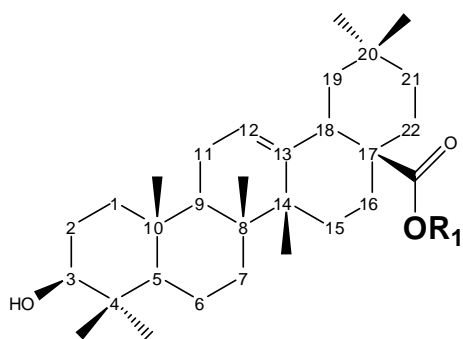


Ginsenoside	R1	R2
Notoginsenoside R1	Glc ² -Xyl	Glc
Ginsenoside Rg1	Glc	Glc
Ginsenoside Re	Glc ² -Rha	Glc
Ginsenoside Rf	Glc ² -Glc	H
Ginsenoside Rh1(20s)	Glc	H
Ginsenoside Rg2(20s)	Glc ² -Rha	Glc
Ginsenoside Rg2(20r)	Glc ² -Rha	Glc
Ginsenoside F1	H	Glc

Ginsenoside	R1
Ginsenoside Rg6	Glc ² -Rha
Ginsenoside Rk3	Glc

Ginsenoside	R1
Ginsenoside F4	Glc ² -Rha
Ginsenoside Rh4	Glc

Fig.2 Structures of protopanaxatriol type aglycone moieties and selected structure of ginsenosides. Glc, β -D-glucose; Rha, α -L-rhamnose; Xyl, β -D-xylose



Ginsenoside	R1
Ginsenoside Ro	Glc ² -Xyl

Fig.3 Structures of oleanae-type aglycone moieties and selected structure of ginsenosides.

Glc, β -D-glucose; Xyl, β -D-xylose

It is proposed that these effects are probably due to their chemical structural similarity with triterpenoid steroid hormones and their amphiphilic nature being able to intercalate into plasma membranes (Christensen LP., 2008). This ability can change membrane fluidity and thus affect membrane function (Christensen LP., 2008).

Polyacetylenes, such as panaxynol, panaxydol and panaxytriol, have in recent years been in focus as potential bioactive constituents of ginseng roots. They inhibit acyl-CoA:cholesterol acyltransferase, tumor cell proliferation, platelet function and promote neurite outgrowth and improve scopolamine-induced memory deficit (Choi SJ. *et al.*, 2008).

Additionally, Ginseng contains phenolic compounds featuring antioxidant activities, proteins having radioprotective effects on victims of an atomic air raid and acidic polysaccharides featuring immune controlling activities (Lee SA. *et al.*, 2012).

Due to the fact that ginseng is a very popular phytomedicine used all around the world, a huge quantity of work has been carried out during the last 40 years in order to develop analytical methods for the identification, quantification and quality control of ginsenosides in raw plant material, extracts and marketed product (Fuzzati N. 2004., Oleszek Wa. *et al.*, 2002 and Oleszek Wa. *et al.*, 2006). Thus the analysis of ginsenosides was performed with various analytical methods, such as thin-layer chromatography (TLC) (Sanada S. *et al.*, 1974), gas chromatography (GC) (Bombardelli E. *et al.*, 1980 and Cui JF., 1995), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) (Nishi H. *et al.*, 1996) and near infra-red spectroscopy (NIRS) (Yap Kevin YL. *et al.*, 2007) and enzyme immunoassay (EIA) (Jung DW. *et al.*, 2002, Baek SH. *et al.*, 2012). Among these techniques HPLC is by far the most employed analytical method. Because of its

speediness, sensitivity and adaptability to non-volatile and polar compounds, HPLC is ideal for the analysis of ginsenosides (Fuzzati N., 2004, Oleszek Wa. *et al.*, 2002, Oleszek W. *et al.*, 2006). Another advantage is versatility due to the possibility of using different detection techniques such as ultraviolet detector (UVD), evaporative light scattering detector (ELSD), fluorescence detector (FLD), pulsed amperometric detector (PAD), and mass spectrometry detector (MSD).

In the last two decades, metabolomics are considered important tools to be applied and utilized to understand the biology of an organism (Aihua Z. *et al.*, 2010). Metabolomics is a relatively new field of “omics” research concerned with the high-throughput identification and quantification of small molecule metabolites in the metabolome. And it has emerged as an important tool in many disciplines such as human diseases and nutrition, drug discovery, plant physiology and others (Shyur LF. *et al.*, 2008, Wishart DS., 2008, Cevallos JM. *et al.*, 2009, Bictash M. *et al.*, 2010, Madsen R. *et al.*, 2010, Putri SP. *et al.*, 2013). The metabolome of an organism is a compilation of all of its metabolites. Metabolites are small molecules; polymeric biomolecules, such as polysaccharides, lignin, peptides, proteins, DNA and RNA, are excluded from this category. For this reason, metabolomics is called “a snapshot of an organism”, showing which compounds are present and in what quantities at a given time point.

Analysis of a large number of samples might facilitate the identification of patterns or metabolite markers that are characteristic for a species, a cultivar, a certain stage of development, or conditions, such as disease state, stress, or daily and seasonal changes. Thus the high-throughput global analysis of metabolome is a key factor of this field. For this reason, NMR-based metabolite profiling/metabolomics was first used in pioneering studies for the rapid multi-component analysis of biological samples (Kim HK. *et al.*, 2011). And mass

spectrometry (MS) is currently the most widely applied technology in metabolomics studies (Xiao JF. *et al.*, 2012). This research trend is reflected in the research area of ginseng, the metabolomics research for ginseng have been published a lot. In Dan *et al.* the metabolite profiling of different parts of *panax notoginseng* was carried out, (Dan M. *et al.*, 2008) and metabolic profiling of five *paxax* species had been performed in Xie *et al.* (Xie G. *et al.*, 2008). In Zhang *et al.* metabolomics research was applied for holistic quality evaluation of white and red ginseng (Zhang HM. *et al.*, 2012). And differences in chemical composition of ginseng according to the cultivation ages have been investigated metabolomics research as a tool (Shin YS. *et al.*, 2007, Kang JH. *et al.*, 2008, Lin WN. *et al.*, 2010, Kim NH. *et al.*, 2011, Kim NH. *et al.*, 2012, Yang SO. *et al.*, 2012).

First, the present study was performed to established simultaneous determination method of ginsenosides for metabolomic analysis data confirmation and getting basic information of ginseng samples using ultra performance liquid chromatography (UPLC). Separation of the 30 ginsenosides, optimization of eluent condition of UPLC and method validation was undertaken.

And then, an ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS) based metabolomic approach was developed in different species, steaming process and cultivation period of ginseng, respectively. These were performed in order of decreasing phenotype difference in ginseng because it was hard to discriminate groups having similar phenotypes. The validity of metabolomic approach method was verified by comparison to targeted analysis results of different ginseng species. After verification, the method was applied to discrimination of ginseng with different processing step and different cultivation period.

I. Simultaneous determination of 30 ginsenosides in *Panax ginseng* preparations using UPLC

1. Purpose of the present study

Among the different detection techniques of ginsenoside analysis, HPLC-UV method is the most employed since it is by far the most common detector found in phytochemical laboratories. Thus many research papers were published for simultaneous determination of ginsenosides (Yamaguchi H. *et al.*, 1998, Kanazawa H *et al.*, 1990, Kanazawa H *et al.*, 1993, Chung WC. *et al.*, 1994, Court WA. *et al.*, 1996 and Shi Y. *et al.*, 2010). But the main problems encountered in performing HPLC-UV analyses of ginseng are the high level of baseline noise and the poor sensitivity due to the weak UV absorption of the ginsenosides (Christensen LP. 2008 and Fuzzati N. 2004). Thus many other researchers had been developed the analytical method of ginsenosides using HPLC-ELSD (Park MK. *et al.*, 1996, Kwon SW. *et al.*, 2001, Kim SN. *et al.*, 2007 and Sun BS. *et al.*, 2009). This method is universal, non-specific detector which can provide a stable baseline even with gradient elution. And other detection techniques of HPLC such as FLD and PAD have been established for simultaneous ginsenosides determination (Shangguan D. *et al.*, 2001 and Lee SI. *et al.*, 2012).

In conventional HPLC system, the choice of particle size must be a compromise. The smaller is the particle size, the higher column back-pressure is occurring in the HPLC system. Recently Ultra performance liquid chromatography (UPLC) could be considered to be a new direction of liquid chromatography. UPLC, which utilize silica particles 1.7 μm , makes it possible to perform better separations in short periods of time. And it has the advantages of the fast analysis,

high peak capacity, great resolution and good sensitivity (Novakova L. *et al.*, 2006 and Guan J. *et al.*, 2007)

In this study, a new UPLC method for the simultaneous determination of 30 ginsenosides, namely ginsenoside Ro, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, 20(*S*)-Rg2, 20(*R*)-Rg2, 20(*S*)-Rg3, 20(*R*)-Rg3, 20(*S*)-Rh1, 20(*S*)-Rh2, 20(*R*)-Rh2, F1, F2, F4, Ra1, Rg6, Rh4, Rk3, Rg5, Rk1, Rb3, Rk2, Rh3, Compound-Y, Compound-K and Notoginsenoside R1 in *Panax ginseng* preparations was developed and validated.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals and Reagents

Ginsenoside Rg1, Re, Rf, 20(*S*)-Rh1, Rb1, Rc, Rb2, Rd, 20(*S*)-Rg3 and 20(*R*)-Rg3 standards were purchased from Chromadex Co. (Irvine, USA) and ginsenoside Ro, 20(*S*)-Rg2, 20(*R*)-Rg2, 20(*S*)-Rh2, 20(*R*)-Rh2, F1, F2, F4, Ra1, Rg6, Rh4, Rk3, Rg5, Rk1, Rb3, Rk2, Rh3, Compound-Y, Compound-K and Notoginsenoside R1 standards were obtained from Ambo institute (Seoul, Korea). Ginsenoside Rg6 and F4, Rk3 and Rh4, Rg5 and Rk1, Rk2 and Rh3 are epimer compounds mixture. The concentration of mixture solutions was calculated using its normalization area percent in UPLC chromatogram because of the UV spectrum of the epimer molecules is the same.

Phosphoric acid was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). And HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). For method validation, red ginseng powder (Lot # 1019008, Korea Ginseng Co., Daejeon, Korea) and red ginseng concentrate (Lot # 2019119, Korea Ginseng Co., Daejeon, Korea) were used. And other *Panax ginseng* preparations were obtained from KGC research institute (Daejeon, Korea). All distilled water used in this experiment was purified by Milli-Q gradient system (Millipore, Bedford, MA, USA) and the resistance value was measured as 18 MΩ prior to use.

2.1 Methods

2.2.1 Sample preparation

The sample preparation of red ginseng powders and concentrates were performed as similar manner as our previous studies using ultrasonic cleaner (In G. *et al.*, 2012).

A half gram of red ginseng powder was weighed in a centrifugal tube (15 mL, PP-single use, BioLogix, USA) and shaken vigorously after the addition of 10 mL of 70% MeOH. Extraction was performed in an ultrasonic cleaner (60 Hz, Wiseclean, Korea) for 30 min. After ultrasonic extraction, centrifugal separation (Legand Mach 1.6R, Thermo, Germany) was performed for 10 min, at 3000 rpm. The resulting supernatant solution was filtered (0.2 μ m, acrodisk, USA) and injected into the UPLC system.

In the case of concentrate type samples, two gram of sample was weighed in a beaker, and 15 mL of deionized water was added. After standing at room temperature for 1 hr, the diluted sample was transferred into a 50 mL volumetric flask where the volume was brought up to 50 mL by adding MeOH. Extraction was performed in an ultrasonic cleaner (60 Hz, Wise-clean, Korea) for 30 min. Then, the solution was filtered (0.2 μ m, acrodisk, USA) and injected into the UPLC system.

2.2.2 Chromatographic condition

The instrumental analysis was performed by Waters ACQUITY UPLC system (Waters, Millford, MA, USA) composed of binary solvent manager (BSM), sample

manager (SM) and photo diode array detector (PDA). The chromatographic separation was accomplished on a ACQUITY BEH C18 column (100 × 2.1 mm, 1.7 μm, Waters, Millford, MA, USA). The column temperature was 40°C. The binary gradient elution system consisted of 0.001% phosphoric acid in water (A) and 0.001% phosphoric acid in acetonitrile (B). The separation was achieved using the following gradient program : 0-0.5 min (15% B), 14.5 min (30% B), 15.5 min (32% B), 18.5 min (38% B), 24.0 min (43% B), 27.0 min (55% B), 27.0 -31.0 min (55% B), 35.0 min (70% B), 38.0 min (90% B), 38.1 min (15% B), 38.1-43.0 min (15% B). The flow rate was set 0.6 mL/min and the sample injection volume was 2.0 μL. The 30 ginsenosides were detected by PDA at 203 nm.

2.2.3 Validation of developed method

The method validation was performed in accordance with ICH guideline (Tavernieers I. *et al.*, 2004, Erner J. 2001). The precision and accuracy of the analytical methods were determined by comparison of triplicates at 3 different concentrations using the same sample and conditions.

Calibration curves, limit of detection (LOD) and limit of quantification (LOQ) were obtained as follows. Due to the distinct variation in contents of ginsenosides in red ginseng powders and red ginseng concentrates, individual ginsenoside standard solutions were prepared and diluted with methanol to appropriate concentration for the establishment of calibration curves. Five concentrations of 30 ginsenoside solutions were injected and then the calibration curves were constructed by plotting the peak areas against the concentration of each ginsenosides. The LOD and LOQ under present chromatographic conditions were determined on the basis of response at a signal-to-noise ratio (S/N) of 3 or 10,

respectively.

Precision and accuracy were obtained as follows. Intra- and inter-day variations were chosen to explain the precision of the UPLC method. Red ginseng powder and concentrate were extracted and analyzed as describe in previous section of sample preparation. The intra-day precision was performed by triplicate extraction and analysis on a single day. The inter-day precision was carried out on 3 different days. Variations were expressed by the relative standard deviations (R.S.D.). The recovery test was performed as similar manner as our previous study (In G. *et al.*, 2012). Accurate amounts of crude saponin fraction were added to approximately half gram of red ginseng powder and two grams of red ginseng concentrate then extracted and analyzed as described in sample preparation sections. The average recoveries were calculated by the following formula: recovery (%) = $100 \times (\text{amount found} - \text{original amount}) / \text{amount spiked}$, with R.S.D. (%) = $(\text{S.D.} / \text{mean}) \times 100\%$.

3. Result and discussion

3.1 Optimization of extraction condition

In order to obtain quantitative extraction of the ginsenosides, variables involved in the procedure such as solvent and extraction time were optimized. Ultrasonic extraction was compared with refluxing. As a result of previous studies, ultrasonic extraction was more simple and effective for extraction of ginsenosides (In G. *et al.*, 2012, Ha YW. *et al.*, 2007). Thus ultrasonic extraction was chosen as extraction method. It involved the following experimental factors and corresponding levels: solvent volume (10, 20, 40 times of the material), methanol concentration (50, 70, 100%, v/v), extraction repetitions (1, 2 or 3 times) and extraction time (10, 30 or 60 min). The optimal condition for the extraction of ginseng powders and concentrates were selected and presented in details in sample preparation section. According to statistic analysis theory, methanol concentration was the most important factor in the extract conditions of red ginseng powders and concentrates and 70% was the best concentration for extraction of the investigated ginsenosides.

3.2 Optimization of UPLC-PDA conditions

For the separation of 30 ginsenosides, it is the key to obtain good resolution between 20(*S*)-ginsenoside Rh1 (5) and 20(*S*)-Rg2 (6), ginsenoside Rk3 (17) and F4 (18), as well as ginsenoside Rg5 (26) and 20(*R*)-Rh2 (27), which are the main factors increasing the running time of UPLC. With the great resolution of UPLC, the problem was resolved under appropriate gradient elution and flow rate.

Higher column temperature was used so as to decrease the pressure at higher flow rate, which could improve the resolution and peak shape. Finally, under the optimized UPLC conditions, the resolutions between 20(*S*)-ginsenoside Rh₁ (5) and 20(*S*)-Rg2 (6), ginsenoside Rk3 (17) and F4 (18), as well as ginsenoside Rg5 (26) and 20(*R*)-Rh2 (27) were 1.25, 1.18 and 1.43 respectively. As shown in Fig.4, the investigated ginsenosides were well separated within 35 min for standard solution. The peaks were identified by comparing the retention times of the peaks with those of the individual standard solutions under the same conditions.

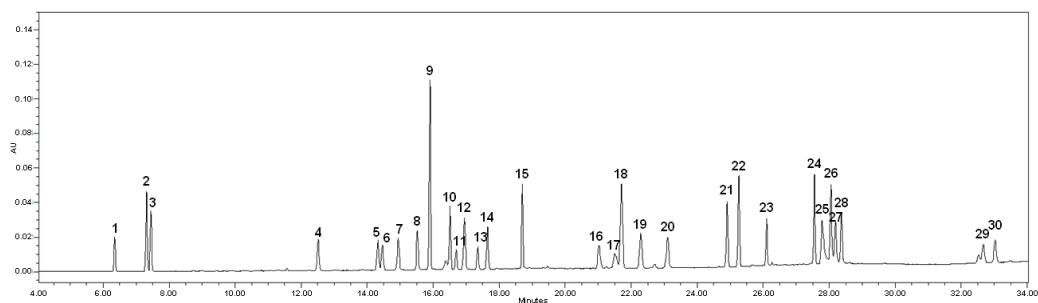


Fig.4 Standard chromatogram of 30 ginsenosides by UPLC. NG-R1; 1, G-Rg1; 2, G-Re; 3, G-Rf; 4, 20(*S*)-G-Rh1; 5, 20(*S*)-G-Rg2; 6, 20(*R*)-G-Rg2; 7, G-Ro; 8, G-Rb1; 9, G-Rc; 10, G-Ra1; 11, G-F1; 12, G-Rb2; 13, G-Rb3; 14, G-Rd; 15, G-Rg6; 16, G-Rk3; 17, G-F4; 18, G-Rh4; 19, G-F2; 20, 20(*S*)-G-Rg3; 21, 20(*R*)-G-Rg3; 22, C-Y; 23, C-K; 24, G-Rk1; 25, G-Rg5; 26, 20(*S*)-G-Rh2; 27, 20(*R*)-G-Rh2; 28, G-Rk2; 29, G-Rh3; 30.

And specially, in the case of ginsenoside Ro, the acidity of mobile phase was the key factor for quantitative determination. As shown in Fig. 5, in chromatogram of ginsenoside Ro, huge difference observed when phosphoric acid was added or not added as the result between. It can be seen that by using the mobile phase without acid, the peak shape was broadened because of insufficient interaction between analytes and solid sorbent in analytical column. But when small amounts of phosphoric acid were added the peak shape of ginsenoside Ro was dramatically sharpened and more retained. This is due to the fact that the solid sorbents were protonated by added phosphoric acid and thus this phenomenon made better interactions between ginsenosides Ro and solid sorbents in analytical column. And the important of acidity of mobile phase was emphasized by other previous studies and these results were in good agreement (Kanazawa H. *et al.*, 1993, Chung WC. *et al.*, 1994 and Court WA. *et al.*, 1996).

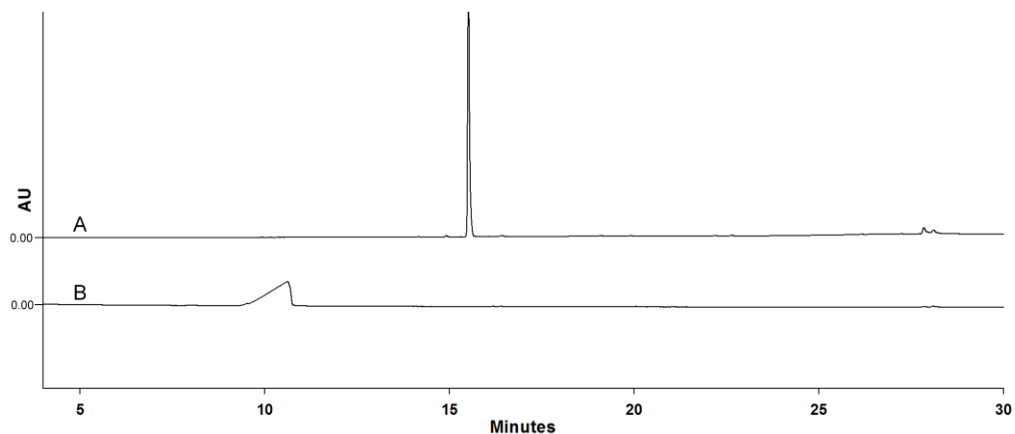


Fig. 5 Effect of acid on the chromatogram of ginsenosides Ro in UPLC system. In the presence of 0.001% phosphoric acid (A), in the absence of phosphoric acid (B)

And thus we investigate the following experimental factors such as type of acid and concentration of acid for simultaneous analysis of 30 ginsenosides. As shown in Fig. 6, in the case of formic acid, the signal to noise ratio was higher and baseline drift was deeper with an increase in the concentration. Therefore, the optimum solvent system was selected as adding phosphoric acid to the mobile phases to the concentration of 0.001%.

3.3 Validation of the developed method

The specificity of individual ginsenosides was confirmed by demonstrating a sufficient separation of the substance present in the sample matrix. As shown in Fig.4 and Fig.7, sample chromatogram compared with that of the standard solution was sufficient to confirm the specificity of ginsenosides. In other words, appropriate separation means that there is adequate resolution between the analyte peaks and the impurity and placebo peaks that need not be separated from each other (Erner J. 2001).

A linear dependence of the signal and the analyte concentration is the most convenient indicator of sample quality or purity and is widely used in pharmaceutical analysis (Erner J. 2001). The standard solutions of each ginsenoside were injected into the UPLC and calibration curves were plotted as the peak area versus the amount of each ginsenoside. The linearity was evaluated by linear regression analysis, which is calculated by the least squares regression method. The LOD and LOQ under the present chromatographic conditions was determined on the basis of the response at a signal to noise ratio of 3 and 10, respectively. And these data were summarized in Table 2.

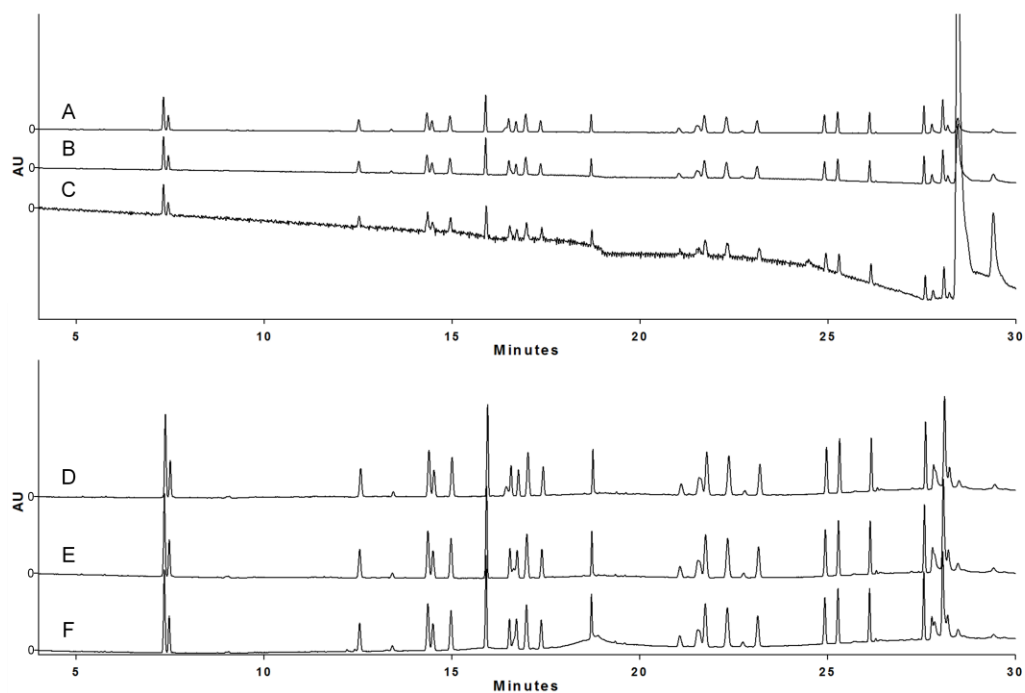


Fig.6 Change of ginsenoside standard chromatogram depending on a type of acids and its concentration. 0.001% formic acid (A), 0.01% formic acid (B), 0.1% formic acid (C), 0.001% phosphoric acid (D), 0.01% phosphoric acid (E), 0.1% phosphoric acid (F).

Table.2 Linearity of calibration curves for 30 ginsenosides

Analytes	Linear regression data			LOD (mg/L)	LOQ (mg/L)
	Calibration curve	r ²	Test range(mg/L)		
NG-R1	$Y = 1.03 \times 10^3 X + 4.89 \times 10^3$	0.999853	9.1 – 914	0.94	3.12
G-Rg1	$Y = 4.52 \times 10^3 X - 4.25 \times 10^3$	0.999933	11.2 – 1120	1.74	5.80
G-Re	$Y = 3.82 \times 10^3 X + 8.88 \times 10^2$	0.999961	3.1 – 306	1.72	5.70
G-Rf	$Y = 3.92 \times 10^3 X - 9.62 \times 10^2$	0.999958	4.5 – 448	1.08	3.59
20(S)-G-Rh1	$Y = 6.45 \times 10^3 X - 7.48 \times 10^3$	0.99997	5.0 – 504	0.85	2.82
20(S)-G-Rg2	$Y = 5.03 \times 10^3 X - 1.68 \times 10^4$	0.999932	3.9 – 387	0.36	1.21
20(R)-G-Rg2	$Y = 5.30 \times 10^3 X + 1.16 \times 10^4$	0.999978	4.0 – 398	0.48	1.58
G-Ro	$Y = 1.08 \times 10^3 X + 2.37 \times 10^3$	0.999928	10.1 – 1010	1.18	3.94
G-Rb1	$Y = 3.33 \times 10^3 X - 4.70 \times 10^3$	0.999937	11.0 – 1104	0.89	2.97
G-Rc	$Y = 3.69 \times 10^3 X - 1.12 \times 10^4$	0.999937	3.7 – 366	1.91	6.37
G-Ra1	$Y = 7.68 \times 10^3 X + 3.83 \times 10^3$	0.999841	8.2 – 824	1.33	4.43
G-F1	$Y = 1.44 \times 10^3 X + 5.22 \times 10^3$	0.999903	8.5 – 852	0.98	3.26
G-Rb2	$Y = 3.55 \times 10^3 X - 5.54 \times 10^3$	0.999937	4.0 – 400	1.76	5.86
G-Rb3	$Y = 7.64 \times 10^3 X + 7.07 \times 10^3$	0.999653	11.0 – 1100	1.67	5.56
G-Rd	$Y = 4.12 \times 10^3 X - 5.72 \times 10^3$	0.999924	3.4 – 340	1.46	4.85
G-Rg6	$Y = 2.33 \times 10^3 X - 5.34 \times 10^2$	0.9999	1.9 – 191	0.80	2.67
G-Rk3	$Y = 3.48 \times 10^3 X + 1.10 \times 10^4$	0.999846	4.2 – 422	0.72	2.42
G-F4	$Y = 2.26 \times 10^3 X - 9.96 \times 10^2$	0.999939	6.6 – 655	0.75	2.51
G-Rh4	$Y = 3.47 \times 10^3 X + 1.70 \times 10^4$	0.999827	6.0 – 603	0.47	1.58
G-F2	$Y = 1.18 \times 10^3 X - 1.05 \times 10^3$	0.999978	6.2 – 624	1.26	4.21
20(S)-G-Rg3	$Y = 4.18 \times 10^3 X - 3.41 \times 10^3$	0.99991	3.3 – 385	0.34	1.12
20(R)-G-Rg3	$Y = 5.71 \times 10^3 X - 6.03 \times 10^3$	0.999919	3.9 – 386	0.20	0.66
C-Y	$Y = 1.25 \times 10^3 X + 3.28 \times 10^3$	0.999865	8.2 – 816	0.76	2.54
C-K	$Y = 1.46 \times 10^3 X + 3.42 \times 10^3$	0.999964	8.5 – 853	0.56	1.88
G-Rk1	$Y = 3.17 \times 10^3 X + 2.56 \times 10^4$	0.999706	4.3 – 427	0.31	1.04
G-Rg5	$Y = 2.77 \times 10^3 X - 2.35 \times 10^4$	0.996517	1.5 – 149	1.16	3.87
20(S)-G-Rh2	$Y = 2.62 \times 10^3 X + 4.37 \times 10^3$	0.999924	3.8 – 383	0.13	0.43
20(R)-G-Rh2	$Y = 8.51 \times 10^3 X + 1.23 \times 10^2$	0.999939	3.6 – 360	0.04	1.14
G-Rk2	$Y = 2.96 \times 10^3 X + 4.48 \times 10^4$	0.996686	6.6 – 492	0.36	1.19
G-Rh3	$Y = 2.94 \times 10^3 X + 2.70 \times 10^4$	0.996538	3.8 – 284	0.34	1.15

NG : notoginsenoside ; G : ginsenoside ; C: compound

The precision of the developed UPLC method was determined by intra- and inter-day variations. Red ginseng and red ginseng concentrate sample were extracted and analyzed as materials and methods sections. Table 3 shows a summary of intra- and inter-day precision. The intra- and inter- day precision (relative standard deviation, R.S.D.) of red ginseng powder was ranged from 1.8 to 10.2 at three different sample amounts. However, in the case of red ginseng concentrate, the intra- and inter-day variations tended to be a little too big for ginsenoside Rk1 and ginsenoside Rg5.

The accuracy of the developed method was tested by spiking experiments for recovery investigations. Thus crude saponin fractions were spiked into the analytical samples for recovery tests of the 30 ginsenosides. As shown in Table 3, the recovery value of red ginseng powders and concentrate were ranged from 89 to 108% and from 98 to 118%, respectively. However, with regards to the precision and accuracy experiments, it should be confirmed that the developed method was suitable for simultaneous determination of ginsenosides in red ginseng and concentrate.

Table.3 Intra- and inter-day variations of the UPLC-PDA method for determination of 30 ginsenosides (n=3)

Analytes	Red ginseng powders				Red ginseng concentrates			
	Intra-day precision		Inter-day precision		Intra-day precision		Inter-day precision	
	content (mg/g)	R.S.D. (%)	content (mg/g)	R.S.D. (%)	content (mg/g)	R.S.D. (%)	content (mg/g)	R.S.D. (%)
NG-R1	0.16	2.30	0.16	2.28	0.13	2.40	0.13	3.69
G-Rg1	2.96	1.89	2.95	1.92	2.26	1.11	2.47	8.84
G-Re	1.96	1.94	1.95	1.95	2.40	2.82	2.58	7.50
G-Rf	0.76	1.79	0.76	2.04	1.04	1.27	1.11	6.78
20(S)-G-Rh1	0.25	1.96	0.25	1.99	0.81	0.80	0.88	7.26
20(S)-G-Rg2	0.27	2.82	0.27	2.62	0.90	0.89	0.96	7.02
20(R)-G-Rg2	0.08	4.63	0.08	4.00	0.44	2.96	0.45	4.01
G-Ro	0.91	2.38	0.92	3.92	1.23	3.34	1.25	4.68
G-Rb1	5.47	3.30	5.46	3.33	8.90	0.57	9.57	7.26
G-Rc	1.38	3.79	1.38	3.92	2.67	1.18	2.85	6.61
G-Ra1	1.63	2.63	1.63	2.67	3.19	1.39	3.24	2.17
G-F1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
G-Rb2	2.33	2.51	2.32	2.74	3.81	0.58	3.77	7.73
G-Rb3	0.38	2.44	0.37	2.48	0.62	0.85	0.62	2.11
G-Rd	0.46	3.24	0.46	3.41	0.94	0.77	1.02	7.54
G-Rg6	0.06	2.51	0.06	2.71	0.26	0.96	0.26	1.55
G-Rk3	0.07	3.30	0.07	2.68	0.16	2.99	0.16	3.36
G-F4	0.11	2.71	0.11	3.16	0.53	1.31	0.52	1.77
G-Rh4	0.11	2.69	0.11	3.04	0.31	1.36	0.31	1.68
G-F2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20(S)-G-Rg3	0.15	5.53	0.15	6.04	0.88	1.92	0.95	7.64
20(R)-G-Rg3	0.05	8.81	0.05	10.19	0.43	8.29	0.46	9.99
C-Y	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C-K	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
G-Rk1	0.05	2.78	0.05	2.88	0.31	24.98	0.33	32.17
G-Rg5	0.05	6.06	0.05	6.48	0.57	20.59	0.58	20.43
20(S)-G-Rh2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20(R)-G-Rh2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
G-Rk2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
G-Rh3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

NG : notoginsenoside ; G : ginsenoside ; C: compound ; N.D. : not detected

Table.4 Accuracy of UPLC-PDA method for the determination of 30 ginsenosides (n=3)

Analytes	Red ginseng powder					Red ginseng concentrate				
	Original mg	Spiked mg	Found mg	Recovery (%)	R.S.D. (%)	Original mg	Spiked mg	Found mg	Recovery (%)	R.S.D. (%)
NG-R1	ND	ND	ND	ND	ND	0.05	0.03	0.07	84.8	16.18
G-Rg1	1.33	1.24	2.56	99.1	1.74	1.03	1.48	2.47	97.4	0.67
G-Re	0.90	1.40	2.28	98.5	1.65	1.09	1.70	2.72	96.1	0.72
G-Rf	0.35	0.84	1.16	97.0	1.12	0.46	0.98	1.40	95.8	0.70
20(S)-G-Rh1	0.11	0.66	0.75	97.3	0.65	0.37	0.81	1.10	90.5	4.17
20(S)-G-Rg2	0.11	1.00	1.08	97.5	0.60	0.41	1.33	1.64	92.8	0.66
20(R)-G-Rg2	0.02	0.59	0.66	107.6	3.51	0.23	0.69	0.92	101.0	1.11
G-Ro	0.70	0.40	1.18	121.0	4.71	0.52	0.32	0.80	88.3	6.57
G-Rb1	2.69	5.11	7.63	96.5	0.89	4.02	6.12	9.74	93.4	0.95
G-Rc	0.79	1.52	2.30	99.2	0.80	1.22	1.93	3.05	94.7	0.28
G-Ra1	1.03	2.14	3.19	100.7	1.06	1.36	2.02	3.31	96.0	0.30
G-F1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
G-Rb2	0.96	2.42	3.39	100.8	0.48	1.76	2.90	4.52	94.9	1.02
G-Rb3	0.20	0.45	0.66	101.5	0.69	0.26	0.44	0.68	93.9	0.44
G-Rd	0.22	1.02	1.20	95.5	0.05	0.43	1.24	1.62	96.6	0.49
G-Rg6	0.03	0.53	0.54	96.0	0.98	0.10	0.47	0.56	99.5	1.32
G-Rk3	0.04	0.28	0.31	95.5	0.35	0.06	0.26	0.32	98.8	1.68
G-F4	0.07	1.05	1.07	95.0	0.26	0.21	0.95	1.14	97.2	0.99
G-Rh4	0.07	0.51	0.56	95.1	1.65	0.12	0.46	0.57	96.7	1.37
G-F2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20(S)-G-Rg3	0.08	2.92	2.86	95.1	0.13	0.42	3.56	3.89	97.4	1.26
20(R)-G-Rg3	0.05	1.00	0.95	90.9	0.62	0.25	1.21	1.40	95.1	1.10
C-Y	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C-K	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
G-Rk1	0.04	1.64	1.55	92.0	0.32	0.13	1.63	1.70	96.0	1.37
G-Rg5	0.08	2.92	2.73	90.8	0.91	0.25	2.64	2.81	96.9	1.16
20(S)-G-Rh2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20(R)-G-Rh2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
G-Rk2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
G-Rh3	N.D.	N.D.	N.D.	N.D.	N.D.	0.00	0.03	0.03	111.8	2.27

NG : notoginsenoside ; G : ginsenoside ; C: compound ; N.D. : not detected

3.4 Analysis of 30 ginsenosides in *Panax ginseng* preparations

There are various types of commercial red ginseng products such as red ginseng (powder), concentrates, tonic, tablets, tea and candy, and they are produced by several ginseng companies. Among these, the most representative red ginseng products are red ginseng (powder) and red ginseng concentrates. Thus these two types of samples were analyzed with the newly developed UPLC method because it was assumed that the different types of red ginseng products contain different types of ginsenosides pattern and different contents of total ginsenosides. And to demonstrate the usefulness of proposed method, fermented ginseng extract and cosmetic raw materials were analyzed. The UPLC chromatograms of the various *Panax ginseng* preparations are shown in Fig.7 and the contents of 30 ginsenosides involved in the *Panax ginseng* preparations are represented in Table 5.

Ginsenoside Ro, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, 20(*S*)-Rg2, 20(*R*)-Rg2, 20(*S*)-Rg3, 20(*R*)-Rg3, 20(*S*)-Rh1, F4, Ra1, Rg6, Rh4, Rk3, Rg5, Rk1, Rb3, and Notoginsenoside R1 were found in both red ginseng powder and red ginseng concentrates samples. Furthermore, the red ginseng concentrate contained a substantially higher amount of 20(*S*)-ginsenoside Rg2, 20(*R*)-Rg2, Rg6, Rk3, F4, Rh4, 20(*S*)-Rg3, 20(*R*)-Rg3, Rk1, Rg5 than red ginseng powder. 20(*S*)-ginsenosides Rg3, 20(*R*)-Rg3, Rk1 and Rg5 can be formed by heat processing through glucosyl elimination and epimerization of Rb1 (Christensen LP. 2008, Ha Y W. *et al.*, 2007 and Lee YJ. *et al.*, 2008). And 20(*S*)-ginsenosides Rg2, 20(*R*)-Rg2, Rg6, Rk3, F4, and Rh4 can be formed by deglycosylation process from Rg1 and Re (Christensen LP. 2008, Ha Y W. *et al.*, 2007 and Yamabe N. *et al.*, 2013). Thus these ginsenosides were likely produced during the process of making the concentrates. To make the concentrates, red ginseng roots are boiled and condensed for a long

period of time. In this period, the sugars attached to aglycones can be removed under conditions of high temperature and pressure, producing various transformed ginsenosides such as 20(*S*)-Rg2, 20(*R*)-Rg2, Rg6, Rk3, F4, Rh4, 20(*S*)-Rg3, 20(*R*)-Rg3, Rk1, Rg5. As we known the fact that, compound-Y and compound-K were observed only in fermented ginseng extract sample. And cosmetic raw materials contained high amount of conversion product of ginsenoside such as ginsenoside Rk3, F4, Rh4, Rk3, 20(*S*)-Rg3, 20(*R*)-Rg3, Rk1 and Rg5. Because of this raw material was acid hydrolyzed product from enriched *Panax ginseng* preparations.

In this study, UPLC-PDA conditions were optimized for the quantitative and qualitative determination of 30 ginsenosides. And 30 ginsenosides were determined in various *Panax ginseng* preparations. This developed method is rapid, accurate, and precise and it can simultaneously determine the 30 ginsenosides in various *Panax ginseng* preparations. These results are definitely helpful to quality control of ginseng product and provide a scientific basis for the research for the components that are responsible for pharmacological effects of red ginseng and related products. And this method may be helpful in the development of new functional materials for cosmetics and natural drugs using other parts of ginseng such as leaf, flower, seed and berry because of its wide range of applications due to the simultaneous analysis of many kinds of ginsenosides.

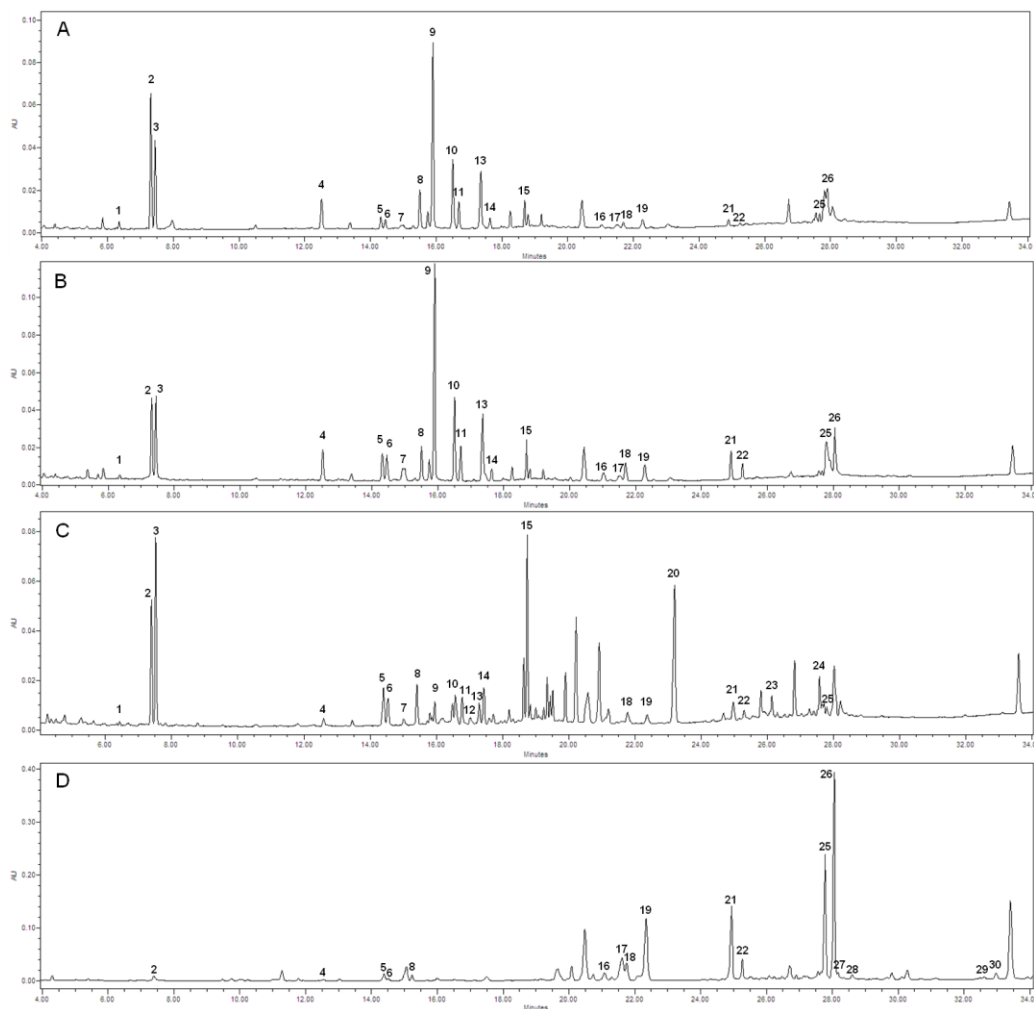


Fig.7 Representative UPLC chromatograms of various *Panax ginseng* preparations. Red ginseng powder (A), red ginseng concentrate (B), Fermented ginseng extract (C), cosmetic raw materials (D) NG-R1; 1, G-Rg1; 2, G-Re; 3, G-Rf; 4, 20(S)-G-Rh1; 5, 20(S)-G-Rg2; 6, 20(R)-G-Rg2; 7, G-Ro; 8, G-Rb1; 9, G-Rc; 10, G-Ra1; 11, G-F1; 12, G-Rb2; 13, G-Rb3; 14, G-Rd; 15, G-Rg6; 16, G-Rk3; 17, G-F4; 18, G-Rh4; 19, G-F2; 20, 20(S)-G-Rg3; 21, 20(R)-G-Rg3; 22, C-Y; 23, C-K; 24, G-Rk1; 25, G-Rg5; 26, 20(S)-G-Rh2; 27, 20(R)-G-Rh2; 28, G-Rk2; 29, G-Rh3; 30.

Table.5 The contents of 30 ginsenosides in various *Panax ginseng* preparations (n=3)

Analytes	Red ginseng powder	Red ginseng concentrate	Fermented ginseng extract	Cosmetic Raw materials
NG-R1	0.153 ± 0.004	0.125 ± 0.003	0.030 ± 0.002	N.D.
G-Rg1	2.886 ± 0.068	2.245 ± 0.017	1.057 ± 0.083	7.118 ± 0.117
G-Re	1.920 ± 0.045	2.361 ± 0.026	1.577 ± 0.126	N.D.
G-Rf	0.745 ± 0.016	1.028 ± 0.006	0.067 ± 0.003	1.746 ± 0.157
20(S)-G-Rh1	0.241 ± 0.007	0.813 ± 0.004	0.334 ± 0.027	7.270 ± 0.455
20(S)-G-Rg2	0.272 ± 0.010	0.897 ± 0.007	0.261 ± 0.021	2.092 ± 0.455
20(R)-G-Rg2	0.076 ± 0.004	0.442 ± 0.010	0.067 ± 0.005	N.D.
G-Ro	0.914 ± 0.052	1.248 ± 0.013	0.384 ± 0.030	5.432 ± 0.282
G-Rb1	5.222 ± 0.166	8.901 ± 0.040	0.204 ± 0.016	N. D.
G-Rc	1.346 ± 0.038	2.682 ± 0.020	0.267 ± 0.022	N. D.
G-Ra1	1.593 ± 0.049	3.214 ± 0.026	0.633 ± 0.055	N. D.
G-F1	N.D.	N.D.	0.043 ± 0.003	N. D.
G-Rb2	2.260 ± 0.068	3.816 ± 0.023	0.458 ± 0.037	N. D.
G-Rb3	0.368 ± 0.009	0.619 ± 0.004	0.112 ± 0.022	N. D.
G-Rd	0.448 ± 0.014	0.949 ± 0.004	1.230 ± 0.096	N. D.
G-Rg6	0.063 ± 0.001	0.264 ± 0.002	N.D.	6.940 ± 0.716
G-Rk3	0.067 ± 0.001	0.163 ± 0.003	N.D.	15.643 ± 0.208
G-F4	0.110 ± 0.002	0.535 ± 0.003	0.073 ± 0.005	13.042 ± 1.302
G-Rh4	0.109 ± 0.004	0.313 ± 0.003	0.046 ± 0.003	37.570 ± 0.763
G-F2	N.D.	N.D.	1.929 ± 0.138	N.D.
20(S)-G-Rg3	0.144 ± 0.006	0.884 ± 0.005	0.189 ± 0.011	80.291 ± 2.569
20(R)-G-Rg3	0.045 ± 0.003	0.442 ± 0.005	0.061 ± 0.003	17.957 ± 0.837
C-Y	N.D.	N.D.	0.107 ± 0.007	N.D.
C-K	N.D.	N.D.	0.169 ± 0.009	N.D.
G-Rk1	0.051 ± 0.004	0.296 ± 0.002	0.023 ± 0.001	43.184 ± 0.815
G-Rg5	0.051 ± 0.004	0.569 ± 0.006	N.D.	61.347 ± 0.773
20(S)-G-Rh2	N.D.	N.D.	N.D.	1.158 ± 0.107
20(R)-G-Rh2	N.D.	N.D.	N.D.	0.332 ± 0.005
G-Rk2	N.D.	N.D.	N.D.	3.923 ± 0.195
G-Rh3	N.D.	N.D.	N.D.	0.969 ± 0.027

NG : notoginsenoside ; G : ginsenoside ; C: compound ; N.D. : not detected

II. Metabolomic approach for the discrimination of processed ginseng species (*Panax ginseng* and *Panax quinquefolius*)

1. Purpose of the present study

Ginseng has been considered one of the most valuable medicinal herbs in oriental countries for over 2000 years, and still it is widely used as an alternative medicine and health-food (Soldati F., 2000). The roots of *Panax ginseng* (Korean ginseng) and *Panax quinquefolius* (American ginseng), two closely related herbal species both belonging to the *Panax* genus, are two of the most commonly used medicinal herbs. However, these two kinds of ginseng genus have been used for different purposes at all in oriental traditional medicine. Therefore, the discrimination and differentiation of these two analogous herbal genera are important in terms of food safety and pharmaceutical efficacy. The morphology and chemical compositions of *Panax ginseng* and *Panax quinquefolius* are very similar, so the traditional methods based on morphological and physicochemical characteristics for identification of these two species are not easy to learn or use. The study of the currently known most reliable method was based on chromatographic separation of isomeric compounds of ginsenoside Rf and 24(R)-pseudoginsenoside F11; two potential markers present in *Panax ginseng* and *Panax quinquefolius* (Chang YS. *et al.*, 2003, Chan TWD. *et al.*, 2000, Leung Kelvin SY. *et al.*, 2007).

In this study, an ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-ToF MS) based metabolomic approach was developed to differentiate between processed *Panax ginseng* (red ginseng) and processed *Panax quinquefolius* (red ginseng). This non-targeted global analysis

method was confirmed by the targeted analysis of ginsenosides, including well-known potential marker substances (ginsenoside Rf and 24(R)-pseudoginsenoside F11).

1. Materials and methods

2.1 Materials

2.1.1 Ginseng samples

Processed *Panax ginseng* (good grade red ginseng, 20 roots per 600 g size) was supplied by the Korea Ginseng Corporation (Daejeon, Korea). Processed *Panax quinquefolius* (cultivated red, large size, 20 roots) was purchased from Hsu's Ginseng Enterprises, Inc. (Marathon County, Wisconsin, US. [http : //www.hsuginseng.com](http://www.hsuginseng.com)).

2.1.2 Chemicals and Reagents

Ginsenoside Rg1, Re, Rf, 20(*S*)-Rh1, Rb1, Rc, Rb2, Rd, 20(*S*)-Rg3 and 20(*R*)-Rg3 standards were purchased from Chromadex (Irvine, CA, USA) and ginsenoside Ro, 20(*S*)-Rg2, 20(*R*)-Rg2, 20(*S*)-Rh2, 20(*R*)-Rh2, F2, F4, Ra1, Rg6, Rh4, Rk3, Rg5, Rk1, Rb3, Rk2, Rh3, notoginsenoside R1, 24(*R*)-pseudoginsenoside F11 and gypenoside XVII standards were obtained from the Ambo Institute (Seoul, Korea). Phosphoric acid was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). Formic acid (eluent additive for LC-MS) was purchased from Fluka (St. Louis, US). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All distilled water used in this experiment was purified by the Milli-Q gradient system (Millipore, Bedford, MA, USA) and the resistance value was measured as 18 M Ω prior to use.

2.2 Methods

2.2.1 Sample preparation

The sample preparation was performed in a similar manner to our previous studies using the ultrasonic extraction method (In G. *et al.*, 2012, and Park H-W. *et al.*, 2013). A half gram of the dried and grounded processed ginseng sample was weighed in a centrifugal tube (15 mL, PP-single use; BioLogix Group, Jinan, Shandong, China) and shaken vigorously after the addition of 10 mL of 50% methanol. Then the extraction was placed in an ultrasonic cleaner (60 Hz; Wiseclean, Seoul, Korea) for 30 min. The solution was centrifuged (Legand Mach 1.6R; Thermo, Frankfurt, Germany) at a speed of 3000 rate/min for 10 min and the aliquot of the supernatant solution was filtered (0.2 µm; Acrodisk, Gelman Sciences, Ann Arbor, MI, USA) and injected into the UPLC system (Waters Co., Milford, MA, USA).

2.2.2 Liquid chromatography

The instrumental analysis was performed by Ultra Performance Liquid Chromatography (UPLC) using an ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 µm; Waters Co., Milford, MA, USA) on Waters ACQUITY UPLC system with a binary solvent manager, sample manager and photo diode array detector. The column temperature was 40°C. The binary gradient elution system consisted of 0.001% phosphoric acid in water (A) and 0.001% phosphoric acid in acetonitrile (B). The separation was achieved using the following protocol: 0-0.5 min (15% B), 14.5 min (30% B), 15.5 min (32% B), 18.5 min (38% B), 24.0 min (43% B),

27.0 min (55% B), 27.0 -31.0 min (55% B), 35.0 min (70% B), 38.0 min (90% B), 38.1 min (15% B), 38.1-43.0 min (15% B). The flow rate was set 0.6 mL/min and the sample injection volume was 2.0 μ L. The individual ginsenosides in the eluents were determined at a UV wavelength of 203 nm using a Photo Diode Array detector (PDA).

2.2.3 Mass spectrometry

Metabolite profiling of *Panax ginseng* and *Panax quinquefolius* was performed by coupling a Waters ACQUITY UPLC system to a Waters Xevo QTOF mass spectrometer (Waters MS Technologies, Manchester, UK) with electrospray ionization (ESI) interface. The source and desolvation gas temperatures were kept at 400 and 120 °C, respectively. The nebulizer and desolvation gas used was N₂ gas. The flow rates of the nebulizer gas and cone gas were set at 800 and 50 L/h, respectively. The capillary and cone voltages were adjusted to 2300 and 40 V, separately. The mass accuracy and reproducibility were maintained by infusing lockmass (leucine-enkephalin, 200 pg/L) thorough LocksprayTM at a flow rate of 20 μ L/min. Centroided data was collected for each sample from 150 to 1,300 Da and the m/z values of all acquired spectra were automatically adjusted during acquisition based on lockmass and dynamic range enhancement. The accurate mass and molecular formula assignments were obtained with MassLynxTM 4.1 software (Waters MS Technologies, Manchester, UK).

2.2.4 Multivariate analysis

To evaluate the potential characteristic components of processed *Panax ginseng* and processed *Panax quinquefolius*, the ESI⁻ raw data of all samples was calculated with MassLynxTM application manager version 4.1 (Waters MS Technologies, Manchester, UK). The method parameters were as follows: the retention time range was 2 to 37 min, the mass range was 150 to 1300 Da, and the mass tolerance was 0.07 Da. The parameters of peak widths at 5% height and peak-to-peak baseline noise were automatically calculated for peak integration. Additionally, the noise elimination level was set to 0.10, and the retention time tolerance was set to 0.2 min. Any specific mass or adduct ions were not excluded, but the isotopic peaks were removed in the multivariate analysis. For data analysis, a list of the intensities of the detected peaks was generated using the pair of retention time (t_R) and mass data (m/z) as the identifier of each peak. A temporary ID was assigned to each of these t_R - m/z pairs for data adjustment that was based on their chromatographic elution order of UPLC. Upon completion, the correct peak intensity data for each t_R - m/z pair for all samples was sorted in a table. Ions from different samples were considered to be the same when they showed the identical t_R and m/z values. MarkerLynxTM (Waters MS Technologies, Manchester, UK) was used for the normalization of each detected peak against the sum of the peak intensities within that sample. The resulting data consisted of a peak number (t_R - m/z pair), sample name and ion intensity. Then the consequent data sets were analyzed by principal component analysis (PCA) and orthogonal partial least squared discriminant analysis (OPLS-DA) using the MarkerLynxTM.

3. Results and discussion

3.1 Targeted analysis

The first step of the experimental procedures employed in this study involved gathering information about a number of the processed ginseng (red ginseng) samples and the confirmation of known biomarkers as noted in the literature (Chang YS. *et al.*, 2003, Chan TWD. *et al.*, 2000, and Leung Kelvin SY. *et al.*, 2007). Therefore, the ginsenosides analysis was performed as a part of the targeted analysis. Also, this ginsenoside analysis was performed in the same manner as in our previous studies (In G. *et al.*, 2012, and Park HW. *et al.*, 2013). The UPLC chromatograms of the processed *Panax ginseng* (Korean red ginseng, KRG) and processed *Panax quinquefolius* (American red ginseng, ARG) are shown in Fig. 8 and the contents of the ginsenosides involved in the two processed ginseng (red ginseng) genera are represented in Table 6. In summary, the ginsenosides Ro, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, 20(*S*)-Rg2, 20(*R*)-Rg2, 20(*S*)-Rg3, 20(*R*)-Rg3, 20(*S*)-Rh1, F4, Ra1, Rg6, Rh4, Rk3, Rg5, Rk1, Rb3, and notoginsenoside R1 were found in the KRG samples; in the case of the ARG samples, the ginsenosides Ro, Rb1, Rb2, Rc, Rd, Re, Rg1, 20(*S*)-Rg2, 20(*R*)-Rg2, 20(*S*)-Rg3, 20(*R*)-Rg3, 20(*S*)-Rh1, F2, F4, Rg6, Rh4, Rk3, Rg5, Rk1, Rb3 and notoginsenoside R1 were found.

The ginsenosides Rf and Ra1 are only present in KRG; whereas the ginsenoside F2 is just found in ARG. These findings were in good agreement with previous reports (Chang YS. *et al.*, 2003, Chan TWD. *et al.*, 2000, and Leung Kelvin SY. *et al.*, 2007 and Christensen LP. 2008). The biomarker of KRG, the ginsenoside Rf, was also confirmed in our results. Additionally, the ginsenosides Ra1 and F2 were found as potential biomarkers of ARG. However, the 24(*R*)-

pseudoginsenoside F11 was not detected in ARG because it does not absorb light at 203 nm.

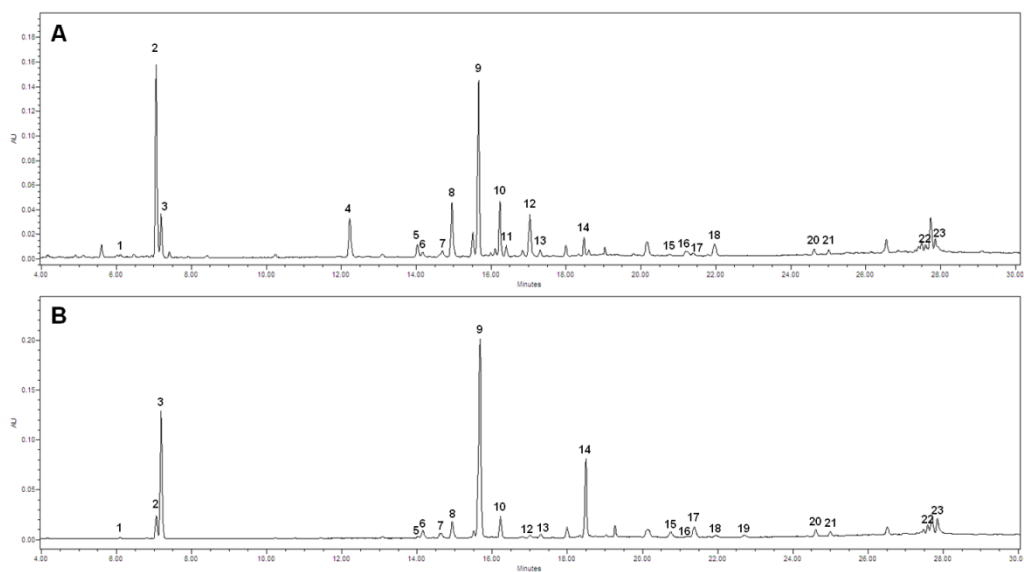


Fig.8 Representative UPLC chromatograms of processed *P. ginseng* (A) and processed *P. quinquefolius* (B). NG-R1; 1, G-Rg1; 2, G-Re; 3, G-Rf; 4, 20(*S*)-G-Rh1; 5, 20(*S*)-G-Rg2; 6, 20(*R*)-G-Rg2; 7, G-Ro; 8, G-Rb1; 9, G-Rc; 10, G-Ra1; 11, G-Rb2; 12, G-Rb3; 13, G-Rd; 14, G-Rg6; 15, G-Rk3; 16, G-F4; 17, G-Rh4; 18, G-F2; 19, 20(*S*)-G-Rg3; 20, 20(*R*)-G-Rg3; 21, G-Rk1; 22, G-Rg5; 23.

Table.6 The contents of ginsenosides in two processed *Panax ginseng* genera (n=20)

Analytes	Processed <i>Panax ginseng</i> (mg/g)	Processed <i>Panax quinquefolius</i> (mg/g)	p value
NG-R1	0.153 ± 0.285	0.009 ± 0.023	3.7x10 ⁻²
G-Rg1	3.339 ± 2.116	0.965 ± 0.584	7.8 x10 ⁻⁵
G-Re	1.518 ± 0.609	6.775 ± 3.621	3.0 x10 ⁻⁶
G-Rf	0.746 ± 0.502	N. D.	2.3 x10 ⁻⁶
20(S)-G-Rh1	0.207 ± 0.138	0.109 ± 0.089	1.2 x10 ⁻²
20(S)-G-Rg2	0.157 ± 0.053	0.827 ± 0.303	4.8 x10 ⁻⁹
20(R)-G-Rg2	0.111 ± 0.071	0.711 ± 0.269	2.2 x10 ⁻⁹
G-Ro	1.640 ± 0.748	2.865 ± 1.392	1.7 x10 ⁻³
G-Rb1	4.700 ± 3.428	26.575 ± 11.936	7.6 x10 ⁻⁸
G-Rc	1.177 ± 0.603	1.021 ± 0.363	3.3 x10 ⁻¹
G-Ra1	0.692 ± 0.725	N. D.	4.2 x10 ⁻⁴
G-Rb2	1.326 ± 0.794	0.146 ± 0.051	2.4 x10 ⁻⁶
G-Rb3	0.218 ± 0.124	0.279 ± 0.104	9.7 x10 ⁻²
G-Rd	0.243 ± 0.204	0.322 ± 1.646	6.5 x10 ⁻⁸
G-Rg6	0.031 ± 0.016	0.486 ± 0.197	3.4 x10 ⁻⁹
G-Rk3	0.058 ± 0.044	0.046 ± 0.031	3.1 x10 ⁻¹
G-F4	0.073 ± 0.036	0.947 ± 0.381	3.7 x10 ⁻⁹
G-Rh4	0.116 ± 0.079	0.092 ± 0.065	3.0 x10 ⁻¹
G-F2	N. D.	0.145 ± 0.158	5.9 x10 ⁻⁴
20(S)-G-Rg3	0.111 ± 0.062	0.937 ± 0.321	3.9 x10 ⁻¹⁰
20(R)-G-Rg3	0.075 ± 0.045	0.597 ± 0.214	6.3 x10 ⁻¹⁰
G-Rk1	0.032 ± 0.023	0.390 ± 0.154	2.0 x10 ⁻⁹
G-Rg5	0.052 ± 0.045	0.894 ± 0.308	1.2 x10 ⁻¹⁰

NG : notoginsenoside ; G : ginsenoside ; N.D. : Not Detected

The contents of the ginsenoside Ra1 in KRG was 0.692 ± 0.725 mg/g and the ginsenoside F2 in ARG was 0.145 ± 0.158 mg/g. For the analysis of these specific ginsenosides, a high level of technology should be supported because of the low contents in ginseng samples and chemical similarity of the ginsenosides' moiety. Therefore, delicate instrumental analysis conditions of a high-resolution value and low background signal are required. These requirements can be fully satisfied by using the UPLC system employed in our previous studies which have been thoroughly studied (Park HW. *et al.*, 2013).

3.2 Non-targeted analysis

To obtain more information about the components of the two processed ginseng species, the UPLC-QToF MS data was used for the non-targeted component analysis. The chromatograms of different kinds of processed ginseng genus were generated with the analysis time of 43 min as in our previous research. The gradient elution mode was employed in the UPLC system to acquire the maximized chromatographic performance including simultaneous data acquisition and appropriate retention times and integration values. This chromatographic data was extracted for multivariate analysis. Fig.9 shows the total ion chromatograms (TIC) of KRG and ARG. The accurate mass measurement was established by the simultaneous but independent acquisition of reference ions of leucine-enkephalin (m/z 556.2771) via the LockSprayTM interface. This system offered several advantages for non-targeted metabolite profiling, including minimization of ion suppression according to the reference ions, and prevention of fluctuations in reference ionization efficiency according to the gradient elution. Using this system, highly improved mass accuracy data was acquired in the range of 0.1 to 20 ppm and the acquired exact mass significantly reduces the number of possible structures of metabolites.

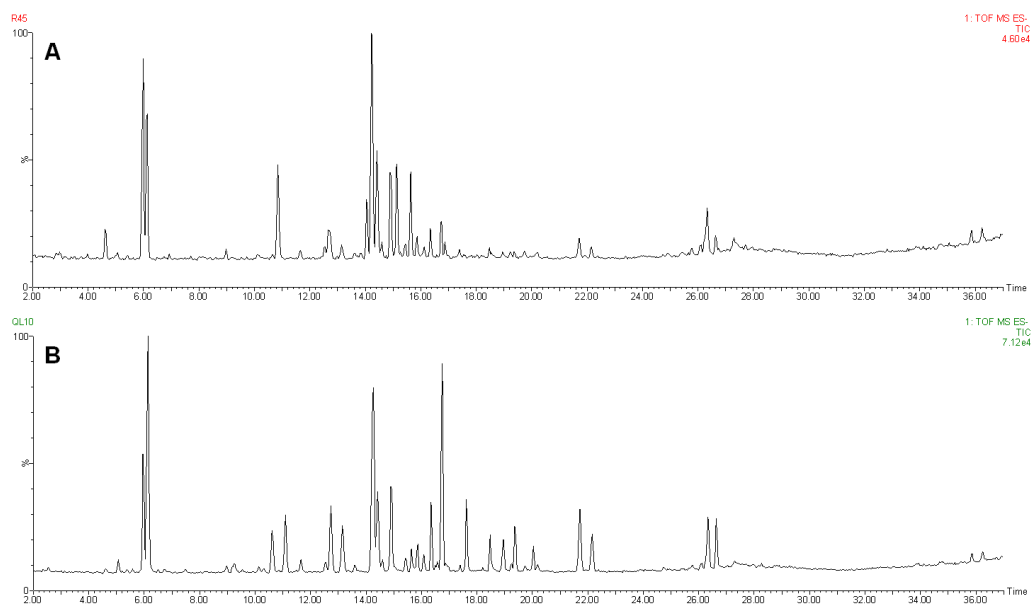


Fig.9 Representative total ion chromatograms (TIC) of ginseng samples. (A) processed *Panax ginseng* (B) processed *Panax quinquefolius*.

In order to find novel discrimination marker ions for KRG and ARG, an unsupervised PCA and supervised OPLS-DA were performed using the UPLC-QTOF MS data. After creating a process for mean-centering and pareto scaled data sets, the data was displayed as score plots (Fig.10). As shown in Fig.10, most KRG and ARG samples were clearly clustered into two groups, i.e., KG and AG groups. This means that the holistic qualities of KRG and ARG were consistent with each other and indeed different in the levels or occurrences of their components.

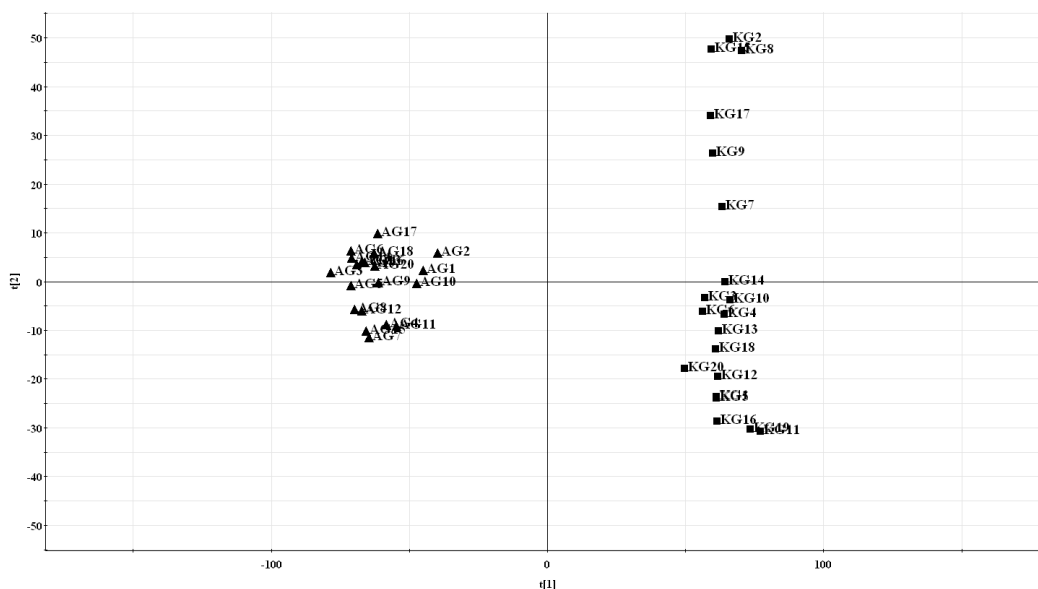


Fig.10 PCA/score plots of processed *Panax ginseng* and processed *Panax quinquefolius* samples using Pareto scaling with mean centering. ■: processed *P. ginseng* (KG) ▲: processed *P. quinquefolius* (AG)

To explore the potential chemical markers that contributed most to the differences between the two groups, UPLC-QTOF MS data from these samples was processed by a supervised OPLS-DA. As shown in Fig. 11A (S-plot), the first six ions, **a** (t_R 16.74 min, m/z 945.5520), **b** (t_R 11.08 min, m/z 799.4848), **c** (t_R 16.74 min, m/z 991.5507), **d** (t_R 6.12 min, m/z 945.5508), **e** (t_R 6.12 min, m/z 991.5513) and **f** (t_R 11.08 min, m/z 845.4691) at the lower left of the “S” were the ions from ARG that contributed most to the differences between the two processed ginseng groups. Analogously as shown in Fig. 11A, six ions, **g** (t_R 15.64 min, m/z 1077.5826), **h** (t_R 10.83 min, m/z 799.4848), **i** (t_R 5.92 min, m/z 845.4995), **j** (t_R 4.61 min, m/z 961.5509), **k** (t_R 15.64 min, m/z 1123.6045) and **l** (t_R 14.90 min, m/z 1077.5825) at the top right corner of the “S” were the ions from KRG that contributed most to the differences between the two groups.

The ion intensity trends of these ions in the tested samples are provided in Fig. 11B. The intensities of ions **b** and **f** were relatively high in all ARG samples, but they were undetectable in the KRG samples. Ions **a**, **c**, **d** and **e** were detected in most of all samples but the intensities of these ions were relatively higher in all of the ARG samples than in the KRG group. The ion intensity trends suggested that components relate to ions **a–f** could be used as potential chemical markers of ARG to distinguish it from KRG. The intensities of ions **h** and **j** were relatively high in all KRG samples but they were undetectable in the ARG. And ions **g**, **i**, **k** and **l** were mainly detected in KRG as relatively higher intensities than in the other group. These ion intensity trends suggest that components related to ions **g–l** could be used as potential chemical markers of KRG to distinguish it from ARG.

In order to identify important potential marker ions, such as ginsenoside Rf, Ra1, F2 and 24(R)- pseudoginsenoside F11, qualitative analysis of the ginsenosides present in KRG and ARG was performed. The identifications of marker ions were confirmed in samples by individual ginsenoside standard materials being compared with respect to each other, and these result summarized in table 7. As a results, ions

b and **f** were the fragment ions from the same molecule, and these ions were $[M-H]^-$ and $[M-H+HCOOH]^-$ from 24(*R*)-pseudoginsenoside F11, respectively. In addition, ion **h** was the $[M-H]^-$ from ginsenoside-Rf. These two ginsenosides occupied important positions on Fig. 11A (top-right and lower-left corner of “S”). This phenomenon was confirmed by the fact that ginsenoside-Rf and 24(*R*)-pseudoginsenoside F11 could be used as marker substances of KRG and ARG, respectively. Ginsenosides Ra1 and F2 were confirmed in all of the samples but do not occupy an important position on Fig. 11A. This is because the ginsenosides Ra1 and F2 had low values of “factor of change” derived from the low concentration and high standard deviation in samples. This means that these ginsenosides showed low contribution to the distinction between the two kinds of processed ginseng genus.

Table.7 Components that were identified from processed *P. ginseng* and processed *P. quinquefolius*

Identity	Chemical Formula	t _R (min)	Mean Measured mass	Theoretical Exact mass	Mass accuracy (ppm)	[M-H] ⁻	[M-H+HCOOH] ⁻
unknown	-	4.61	961.5509	-	-	(j) 961.5509	1007.5630
N-R1	C ₄₇ H ₈₀ O ₁₈	5.06	931.5325	931.5266	6.3	931.5325	977.5453
G-Rg1	C ₄₂ H ₇₂ O ₁₄	5.92	799.4965	799.4844	15.1	799.4965	(i) 845.4995
G-Re	C ₄₈ H ₈₂ O ₁₈	6.12	945.5508	945.5423	8.5	(d) 945.5508	(e) 991.5513
G-Rf	C ₄₂ H ₇₂ O ₁₄	10.83	799.4848	799.4844	0.5	(h) 799.4848	835.5026
pG-F11	C ₄₂ H ₇₂ O ₁₄	11.08	799.4848	799.4844	0.5	(b) 799.4848	(f) 845.4691
G-Rh1	C ₃₆ H ₆₂ O ₉	12.54	637.4426	637.4316	17.3	637.4426	683.4336
20(S)-G-Rg2	C ₄₂ H ₇₂ O ₁₃	12.71	783.4899	783.4895	0.5	783.4899	829.5025
20(R)-G-Rg2	C ₄₂ H ₇₂ O ₁₃	13.16	783.4897	783.4895	0.3	783.4897	829.5046
G-Rb1	C ₅₄ H ₉₂ O ₂₃	14.23	1107.5990	1107.5951	3.5	1107.5990	1153.619
G-Ro	C ₄₈ H ₇₆ O ₁₉	14.41	955.4886	955.4903	-1.8	955.4886	-
G-Rc	C ₅₃ H ₉₀ O ₂₂	14.90	1077.5825	1077.5846	-1.9	(l) 1077.5825	1123.6079
G-Ra1	C ₅₈ H ₉₈ O ₂₆	15.12	1209.6300	1209.6268	2.6	1209.6300	-
G-Rb3	C ₅₃ H ₉₀ O ₂₂	15.86	1077.5988	1077.5846	13.2	1077.5988	1123.6123
G-Rb2	C ₅₃ H ₉₀ O ₂₂	15.64	1077.5826	1077.5846	-1.9	(g) 1077.5826	(k) 1123.6045
G-Rd	C ₄₈ H ₈₂ O ₁₈	16.74	945.5520	945.5423	10.3	(a) 945.5520	(c) 991.5507
Gy-XVII	C ₄₈ H ₈₂ O ₁₈	17.62	945.5453	945.5423	3.2	945.5423	991.5512
G-Rg6	C ₄₂ H ₇₀ O ₁₂	18.93	765.4822	765.4789	4.3	765.4822	811.4964
G-Rk3	C ₃₆ H ₆₀ O ₈	19.23	619.4316	619.4210	17.1	619.4316	665.4370
G-F4	C ₄₂ H ₇₀ O ₁₂	19.38	765.4801	765.4789	1.6	765.4801	811.4951
G-Rh4	C ₃₆ H ₆₀ O ₈	19.73	619.4308	619.4210	15.8	619.4308	665.4356
G-F2	C ₄₂ H ₇₂ O ₁₃	20.03	783.5050	783.4895	16.3	783.5050	829.5000
20(S)-G-Rg3	C ₄₂ H ₇₂ O ₁₃	21.69	783.4882	783.4895	-1.7	783.4882	829.5044
20(R)-G-Rg3	C ₄₂ H ₇₂ O ₁₃	22.16	783.4903	783.4895	1.0	783.4903	829.5070
G-Rk1	C ₄₂ H ₇₀ O ₁₂	26.32	765.4788	765.4789	-0.1	765.4788	-
G-Rg5	C ₄₂ H ₇₀ O ₁₂	26.62	765.4794	765.4789	0.7	765.4794	-
20(S)-G-Rh2	C ₃₆ H ₆₂ O ₈	26.64	-	621.4366	-	-	667.4510
20(R)-G-Rh2	C ₃₆ H ₆₂ O ₈	26.81	-	621.4366	-	-	667.4527
G-Rh3	C ₃₆ H ₆₀ O ₇	29.94	-	603.4261	-	-	649.4399
G-Rk2	C ₃₆ H ₆₀ O ₇	30.41	-	603.4261	-	-	649.4395

NG: notoginsenoside ; G: ginsenoside ; pG: pseudoginsenoside ; Gy: Gypenoside

Other potential marker ions were identified by comparing the spectrums of standards materials and selected ions in samples and individual retention times. Ions **a** and **c** were the fragment ions from the same molecule and these ions were $[M-H]^-$ and $[M-H+HCOOH]^-$ from ginsenoside Rd. Ions **d** and **e** were the fragment ions from ginsenoside-Re with respect to $[M-H]^-$ and $[M-H+HCOOH]^-$. The ions **g** and **k** were confirmed as $[M-H]^-$ and $[M-H+HCOOH]^-$ of ginsenoside Rc and ion **i** was confirmed as the $[M-H]^-$ ion of ginsenoside Rg1 by the use of standard materials. These ions could not be a marker substance, as it is only because of the differences between the concentrations of the two groups is a phenomenon. These are called “false-positive” in metabolomics and should be excluded by other verification methods.

Finally in Fig. 11, ion **j** occupied an important position but it could not be confirmed by standard materials. However, it can be assumed that the ion **j** was thought to be the 20-gluco-ginsenoside Rf, based on exact mass and previous studies (Christensen LP., 2008 and Park J D., 1996)

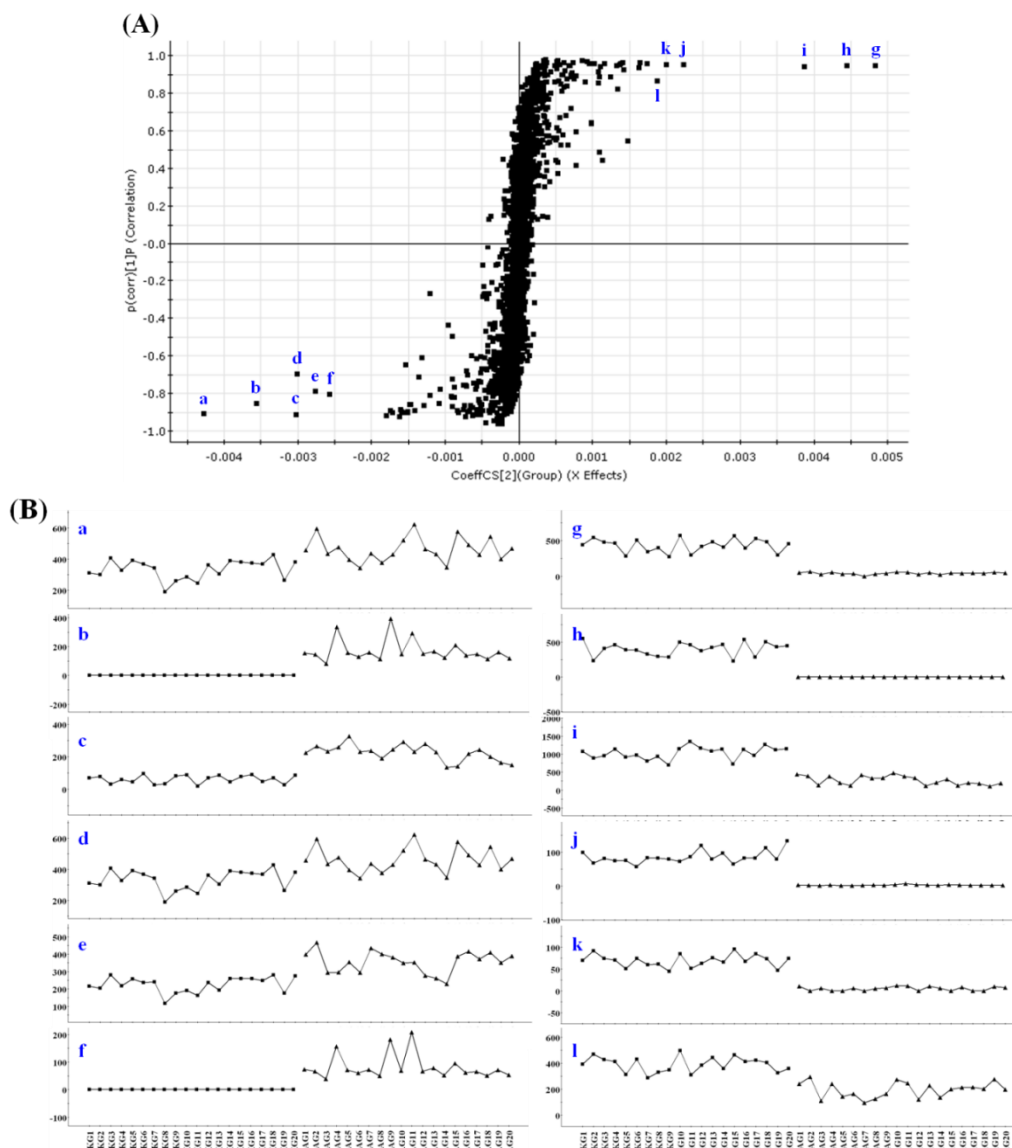


Fig.11 OPLS-DA/S-plots (A) and selected ion intensity trend plots (B) of processed *P. ginseng* (■, KG) and processed *P. quinquefolius* (▲, AG) samples. **a** (t_R 16.74 min, m/z 945.5520), **b** (t_R 11.08 min, m/z 799.4848), **c** (t_R 16.74 min, m/z 991.5507), **d** (t_R 6.12 min, m/z 945.5508), **e** (t_R 6.12 min, m/z 991.5513), **f** (t_R 11.08 min, m/z 845.4691), **g** (t_R 15.64 min, m/z 1077.5826), **h** (t_R 10.83 min, m/z 799.4848), **i** (t_R 5.92 min, m/z 845.4995), **j** (t_R 4.61 min, m/z 961.5509), **k** (t_R 15.64 min, m/z 1123.6045), **l** (t_R 14.90 min, m/z 1077.5825)

III. Metabolomic approach for *in-situ* monitoring of chemical changes in *Panax ginseng* during steaming process

1. Purpose of the present study

In traditional oriental medicines (TOM), the processed medicinal herbs play an important role in the application and its usages. Generally, the main purpose of processing the medicinal herbs is to transform the properties of medicines and to increase the curative effect and reduce or eliminate toxicity and side-effects. The processing methods in TOM have involved special manipulations, such as toasting, steaming, cooking and fermentation. Although ancient practitioners in TOM had been recognized these conversions for therapeutic effects but the change of these constituents has not been fully understood. In recent decades, many processed medicinal herbs have been investigated chemically and pharmacologically but nothing more than piecemeal studies. In the case of ginseng, some processing method was employed and thus some types of commercial ginseng types manufactured by different processing methods after harvest. Fresh ginseng (non-processed ginseng) is rarely used, because it is easily decomposed by high water content (i.e., 70–80%) and coexist soil microorganisms. Therefore, ginseng has been mainly used as two processed form called white ginseng (WG) and red ginseng (RG). WG is produced from peeled or unpeeled fresh ginseng by drying in sunlight and RG is manufactured by steaming the unpeeled fresh ginseng at 95–100 °C for 2–3 h and then drying. However, processing conditions may greatly affected to chemical constituents of ginseng thus there is a considerable differences among types of processed ginseng (Christensen LP., 2008). Therefore many

researchers have been studied the chemical change of ginseng and especially ginsenosides (Lau AJ. *et al.*, 2003, Du XW. *et al.*, 2003, Lau AJ. *et al.*, 2004, Wang D. *et al.*, 2012, Sun BS. *et al.*, 2012, Chu C. *et al.*, 2012 and Xie YY. *et al.*, 2012), phenolics (Jung MY. *et al.*, 2002, and Chung IM. *et al.*, 2012) and amino acids (Cho EJ. *et al.*, 2008). And some research groups have been interested in its biological activity of ginseng and ginsenosides which have been generated during processing times (Park JI. *et al.*, 2002, Kang KS. *et al.*, 2007 Lee YJ. *et al.*, 2008, Sun S. *et al.*, 2010). And recently, chemometric tools, so called “metabolomics” were applied for metabolite profiling and identifying the complicated constituents of steaming induced components and type of ginseng (Toh DF. *et al.*, 2010, and Zhang HM. *et al.*, 2012). Although several studies reported on the chemical change of raw and processed ginseng, there is currently limited sample preparation or sampling; where most studies to date have examined a powdered ginseng were used because of its individual variation between samples.

In this study, *in-situ* monitoring of the steaming induced chemical changes of ginseng was performed. For the *in-situ* analysis of chemical components in ginseng, a pieces of samples divided from the same ginseng root were obtained each processing steps. An ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS) based metabolomic approach was used to monitor of steaming induced chemical changes of *Panax ginseng*. Also, to confirm a result of metabolomic approach, ginsenosides and some maillard reaction products were determined by analytical techniques such as UPLC-PDA and High Pressure Ion Chromatography-Pulsed Amperometric Detector (HPIC-PAD).

2. Materials and methods

2.1 Materials

2.1.1 Ginseng samples

Ginseng Samples (6 year aged *P. ginseng*, 20 roots) were used in this experiment was obtained from red ginseng manufacturing factory of Korea Ginseng Corporation (Buyeo, Chung-nam, Korea) as follows. First, all of samples are given to each of the serial numbers through steaming process because of the sample was able to distinguish each ginseng root. Before the start of steaming process, the washed fresh ginseng (FG,) was made a longitudinal incision to 1/3 was separated and was kept after freeze dried. And then the remaining 2/3 pieces were carried out steaming process in closed chamber as factory setting (98°C, 3 hr). Immediately after steaming process, the steamed ginseng (SG) was sliced 1/3 was separated and kept after freeze dried. And finally, residual 1/3 pieces of ginseng were dried in chamber (65°C, 3 hr) and under day light (13 day) to make red ginseng (RG). Frozen FG and SG samples were freeze-dried and RG were further dried in dry-oven (60°C, 2 hr) and all samples were grinded fine powder and stored in a -20°C freezer until used for analysis.

2.1.2 Chemicals and Reagents

All the reagents were used in these experiments was higher than extra pure grade. And HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Formic acid (eluent additive for LC-MS) was purchased

from Fluka (St. Louis, US). All distilled water used in this experiment was purified by the Milli-Q gradient system (Millipore, Bedford, MA, USA) and the resistance value was measured as 18 M Ω prior to use.

2.2 Methods

2.2.1 Malonyl ginsenosides analysis

Ginsenoside Rg1, Re, Rf, 20(*S*)-Rh1, Rb1, Rc, Rb2, Rd, 20(*S*)-Rg3 and 20(*R*)-Rg3 standards were purchased from Chromadex (Irvine, CA, USA) and ginsenoside 20(*S*)-Rg2, 20(*R*)-Rg2 were obtained from Ambo Institute (Seoul, Korea).

The ginseng sample preparation was performed in a similar manner as in previous studies (Park HW. *et al.*, 2013). And malonyl-ginsenosides were analyzed by indirect base-hydrolysis method as reported (Court WA. *et al.*, 1996). A half gram of ginseng powder was weighed in a centrifugal tube (15 mL, PP-single use; BioLogix Group, Jinan, Shandong, China) and shaken vigorously after the addition of 10 mL of 70% MeOH. Extraction was performed in an ultrasonic cleaner (60 Hz, Wiseclean, Seoul, Korea) for 30 min. After ultrasonic extraction, centrifugal separation (Legand Mach 1.6R; Thermo, Frankfrut, Germany) was performed for 10 min at 3000 rpm. The resulting supernatant solution was filtered (0.2 μ m; Acrodisk, Gelman Sciences, Ann Arbor, MI, USA) and injected into the Ultra Performance Liquid Chromatography (UPLC) system (Waters Co., Milford, MA, USA). And acidic ginsenosides were hydrolysed by adding 80 μ L of 5% KOH to a portion (850 μ L) of the ginseng extract. After 2 h the solution was neutralized by adding 80 μ L of a 0.01 M KH₂PO₄ solution. The mixture was diluted with 850 μ L

of acetonitrile and analyzed to determine total ginsenosides.

The instrumental conditions of UPLC as follows. The chromatographic separation obtained by using an ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 μm; Waters Co., Milford, MA, USA) and the column temperature was 40°C. The binary gradient elution system consisted of 0.01 M KH₂PO₄ in water (A) and acetonitrile (B). The separation was achieved using the following protocol; 0-0.5 min (15% B), 14.5 min (30% B), 15.5 min (32% B), 16.5 min (40% B), 17.0 min (55% B), 21.0 min (90% B), 25.0 -27.0 min (15% B). The flow rate was set 0.6 mL/min and the sample injection volume was 2.0 μL. The ginsenosides were determined at a UV wavelength of 203 nm using a Photo Diode Array detector (PDA).

2.2.2 Amino-sugar analysis

Arginyl-fructose (AF), arginyl-fructose-glucose (AFG) was obtained from Ambo Institute (Seoul, Korea).

A ten miligram of ginseng powder was weighed in a centrifugal tube and shaken vigorously after the addition of 10 mL de-ionized water. Extraction was performed with ultrasonification for 30 min. After ultrasonic extraction, centrifugal separation was performed for 10 min at 3000 rpm. The resulting supernatant solution was filtered with 0.2 μm membrane filter and injected into the ICS-3000 High Pressure Ion Chromatography (HPIC) system (Dionex, Sunnyvale, CA, USA).

The instrumental conditions of HPIC as follows. The chromatographic separation obtained by using a CarboPac PA-1 column (250 mm × 4 mm; Dionex, Sunnyvale, CA, USA) and the column temperature was 30°C. The gradient elution system consisted of 250 mM NaOH (A) and water (B). The separation was

achieved using the following protocol; 0-20 min (93% B), 30-35 min (50% B), 36-45 min (0% B), 46-60 min (93% B). The flow rate was set 1.0 mL/min and the sample injection volume was 5.0 μ L. The analytes were determined using a Pulsed Amperometric Detector (PAD) with Au working electrode and Ag/AgCl reference electrode.

2.2.3 Liquid chromatography and QTOF MS conditions

The instrumental analysis was performed by Ultra Performance Liquid Chromatography (UPLC) using an ACQUITY BEH C18 column (100 mm \times 2.1 mm, 1.7 μ m; Waters Co., Milford, MA, USA) on Waters ACQUITY UPLC system with a binary solvent manager, sample manager and photo diode array detector. The column temperature was 40 °C. The binary gradient elution system consisted of 0.01% formic acid in water (A) and 0.01% formic acid in acetonitrile (B). The separation was achieved using the following protocol: 0-0.5 min (15% B), 14.5 min (30% B), 15.5 min (32% B), 18.5 min (38% B), 24.0 min (43% B), 27.0 min (55% B), 27.0 -31.0 min (55% B), 35.0 min (70% B), 38.0 min (90% B), 38.1 min (15% B), 38.1-43.0 min (15% B). The flow rate was set 0.4 mL/min and the sample injection volume was 2.0 μ L.

Metabolite profiling for *in-situ* monitoring of steaming induced chemical changes *Panax ginseng* was performed by coupling a Waters ACQUITY UPLC system to a Waters Xevo Q-TOF mass spectrometer (Waters MS Technologies, Manchester, UK) with an electrospray ionization (ESI⁻ and ESI⁺) interface. The source and desolvation gas temperatures were kept at 400 and 120 °C, respectively. The nebulizer and desolvation gas used was N₂ gas. The flow rates of the nebulizer gas and cone gas were set at 800 and 50 L/h, respectively. The capillary and cone

voltages were adjusted to 2300 (2200V in ESI⁺) and 40 V(25 V in ESI⁺), separately. The mass accuracy and reproducibility were maintained by infusing lockmass (leucine-enkephalin, 200 pg/L) thorough LocksprayTM at a flow rate of 7 μ L/min. Centroided data was collected for each sample from 100 to 1,450 Da and the m/z values of all acquired spectra were automatically adjusted during acquisition based on lockmass and dynamic range enhancement. The accurate mass and molecular formula assignments were obtained with MassLynxTM 4.1 software (Waters MS Technologies, Manchester, UK).

2.2.4 Multivariate analysis

To evaluate the potential characteristic components of steaming induced chemical change, the ESI⁻ and ESI⁺ raw data of all samples was calculated with MassLynxTM application manager version 4.1 (Waters MS Technologies, Manchester, UK). The method parameters were as follows: the retention time range was 0.3 to 35 min, the mass range was 100 to 1500 Da, and the mass tolerance was 0.1 Da. The parameters of peak widths at 5% height and peak-to-peak baseline noise were automatically calculated for peak integration. Additionally, the noise elimination level was set to 0.10, and the retention time tolerance was set to 0.2 min. Any specific mass or adduct ions were not excluded, but the isotopic peaks were removed in the multivariate analysis. For data analysis, a list of the intensities of the detected peaks was generated using the pair of retention time (t_R) and mass data (m/z) as the identifier of each peak. A temporary ID was assigned to each of these t_R - m/z pairs for data adjustment that was based on their chromatographic elution order of UPLC. Upon completion, the correct peak intensity data for each t_R - m/z pair for all samples was sorted in a table. Ions from different samples were

considered to be the same when they showed the identical t_R and m/z values. MarkerLynxTM (Waters MS Technologies, Manchester, UK) was used for the normalization of each detected peak against the sum of the peak intensities within that sample. The resulting data consisted of a peak number (t_R - m/z pair), sample name and ion intensity. Then the consequent data sets were analyzed by principal component analysis (PCA) and orthogonal partial least squared discriminant analysis (OPLS-DA) using the MarkerLynxTM.

3. Results and discussion

3.1 Non-targeted analysis

At first, UPLC-QTOF MS data was used for the non-targeted component analysis for searching potential marker of chemical changes by steaming process. The chromatograms of each processing step, namely fresh, steamed and red ginseng (FG, SG and RG, respectively), were generated with the analysis time of 43 min as Π -3.2 non-targeted analysis. The gradient elution mode was employed in the UPLC system to acquire the maximized chromatographic performance including simultaneous data acquisition and appropriate retention times and integration values. Addition to ESI-, ESI+ mode was performed to extract more components. Fig. 12 shows the total ion chromatograms (TIC) of FG, SG and RG. The accurate mass measurement was established by the simultaneous but independent acquisition of reference ions of leucine-enkephalin (m/z 554.2615 in ESI⁻ and 556.2771 in ESI⁺) via the LockSprayTM interface. This system offered several advantages for non-targeted metabolite profiling, including minimization of prevention of fluctuations in reference ionization efficiency according to the gradient elution.

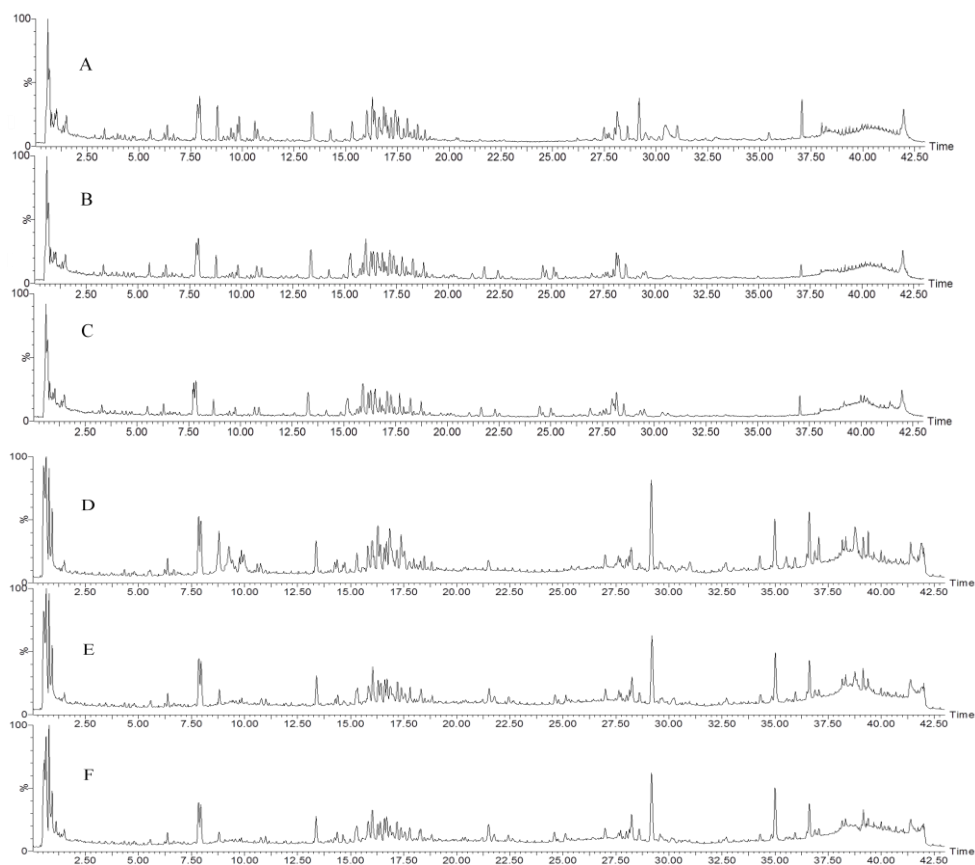


Fig.12 Representative total ion chromatograms of ginseng samples. (A), (D) Fresh ginseng (B), (E) Steamed ginseng (C), (F) Red ginseng ; (A), (B), (C) are ESI mode data and (D), (E), (F) are ESI⁺ mode data

In order to find novel discrimination marker ions for FG, SG and RG an unsupervised PCA and supervised OPLS-DA were performed using the UPLC-QTOF MS data. After creating a process, I could get the results table, displaying the ID, mass and retention time of the marker. Using Ez info software, built in Markerynx, I performed PCA analysis and data was displayed as score plots (Fig. 13). The first principal component, horizontal axis, (PC1) contains the most variance in the data set. The second principal component, (PC2), is orthogonal to PC1, and represents maximum amount of variance not explained by PC1. The remaining components are attained in a similar manner, thereby reducing the high dimensional data sets to a two- or three-dimensional scores map without losing profound information. As shown in Fig. 13, FG, SG and RG samples were clearly clustered into three groups. This means that the holistic qualities of FG, SG and RG were consistent with each other and indeed different in the levels or occurrences of their components.

To explore the potential chemical markers that contributed most to the differences between the groups, UPLC-QTOF MS data from these samples was processed by a supervised OPLS-DA. OPLS-DA is a supervised extension of a PCA and uses class information to maximize the separation between two groups of observation (Yang SO. *et al.*, 2013). As shown in Fig 13, SG group was more close to RG group than FG group. It means that SG group is more similar to RG than FG. So I performed OPLS-DA with FG group and SG/RG group.

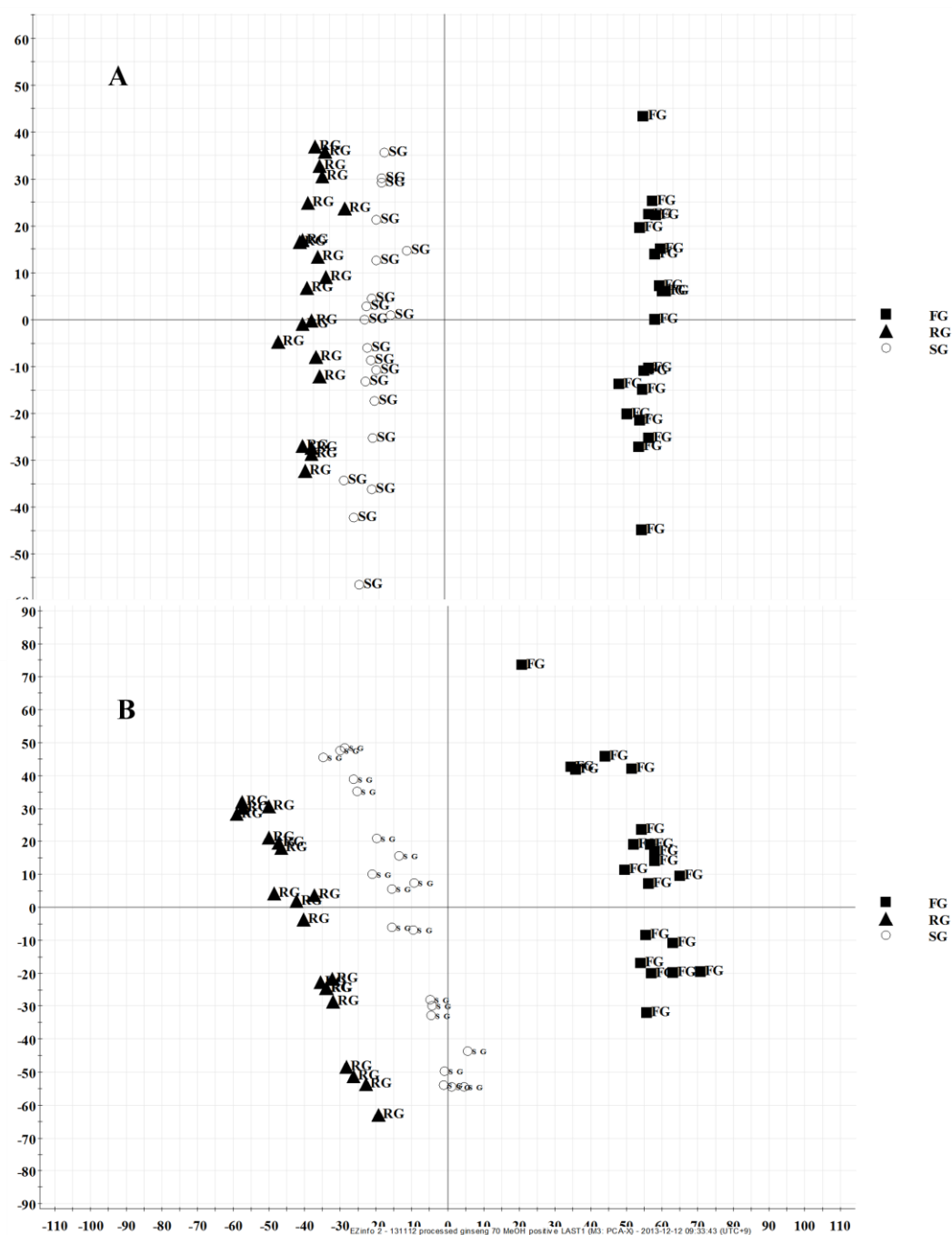


Fig.13 PCA/score plots of fresh ginseng (FG), steamed ginseng (SG) and red ginseng (RG) using Pareto scaling with mean centering (A) ESI⁻ mode data (B) ESI⁺ mode data

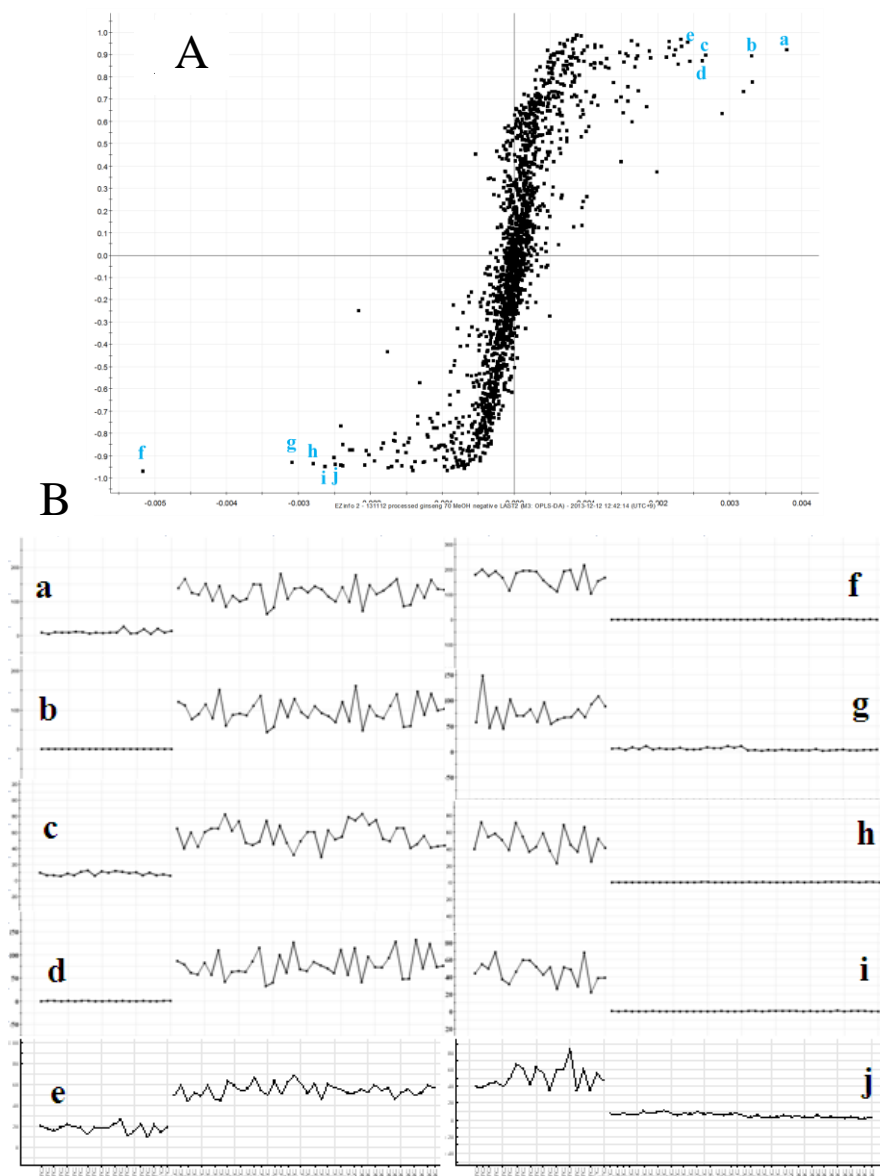


Fig.14 OPLS-DA/S-plots (A) and selected ion intensity trend plots (B) of fresh ginseng (FG), steamed ginseng (SG) and red ginseng (RG) samples from ESI⁻ mode data. **a** (t_R 15.21 min, m/z 683.4259), **b** (t_R 15.85 min, m/z 683.4328), **c** (t_R 0.65 min, m/z 128.0366), **d** (t_R 22.40 min, m/z 665.4231), **e** (t_R 17.77 min, m/z 1149.6014), **f** (t_R 29.16 min, m/z 603.3305), **g** (t_R 10.58 min, m/z 971.4813), **h** (t_R 29.16 min, m/z 649.3427), **i** (t_R 31.00 min, m/z 579.3386), **j** (t_R 0.59 min, m/z 145.0668),

As shown in Fig. 14A and Fig. 15A (S-plot), the first five ions, **a** (t_R 15.21 min, m/z 683.4259), **b** (t_R 15.85 min, m/z 683.4328), **c** (t_R 0.65 min, m/z 128.0366), **d** (t_R 22.40 min, m/z 665.4231), **e** (t_R 17.77 min, m/z 1149.6014), **k** (t_R 0.79 min, m/z 268.1036), **l** (t_R 0.63 min, m/z 268.1036), **m** (t_R 0.65 min, m/z 268.1061), **n** (t_R 0.57 min, m/z 499.1985), **o** (t_R 16.60 min, m/z 425.3700), at the top right corner of the “S” were the ions from SG and RG that contributed most to the differences between FG and SG/RG group. Analogously, **f** (t_R 29.16 min, m/z 603.3305), **g** (t_R 10.58 min, m/z 971.4813), **h** (t_R 29.16 min, m/z 649.3427), **i** (t_R 31.00 min, m/z 579.3386), **j** (t_R 0.59 min, m/z 145.0668), **p** (t_R 0.63 min, m/z 147.1029), **q** (t_R 29.18 min, m/z 425.2822), **r** (t_R 0.65 min, m/z 147.1068), **s** (t_R 29.20 min, m/z 425.2830), **t** (t_R 8.80 min, m/z 423.3549), at the lower left corner of the “S” were the ions from FG that contributed most to the differences between the two groups.

The ion intensity trends of these ions in the tested samples are provided in Fig. 14 and Fig. 15B. The intensities of ions **l** and **n** were relatively high in all SG and RG samples, but they were undetectable in the FG samples. Ions **k**, **m**, **o** and **o** were detected all SG and RG samples but some samples in FG group. And the intensities of these ions were relatively higher in all of the SG and RG samples than in the FG group. The ion intensity trends suggested that components relate to ions **a–e** and **k–o** could be generated in steaming process from FG. The intensities of ions **q** and **s** were relatively high in all FG samples but they were undetectable in the SG and RG. And ions **p**, **r** and **t** were mainly detected in SG and RG group as relatively higher intensities than in the FG group. These ion intensity trends suggest that components related to ions **f–j** and **p–t** could be degraded in steaming process

From comparing the retention time and exact mass with that of reference standards and ions in sample, five ions were identified. Ion **a**, **b**, **d**, **n** and **o** were

identified to ginsenoside Rg2s, Rh1r, Rh4, arginyl-fructose-glucose and ginsenoside Rc, respectively (Fig.16). As shown in table 8, the contents of ginsenoside Rc was not differ considerably. So it was assumed a false positive component. Finally in Fig. 15, ion **e** occupied an important position but it could not be confirmed by standard materials. However, it can be assumed that the ion **e** was thought to be the quinquenoside R1, acetyl-ginsenosides, based on exact mass and previous studies (Geoffrey CK. *et al.*, 2003, Christensen LP.2009). And this acetyl ginsenoside were assumed to be generated from the malonyl-ginsenosides through decarboxylation, hydrolysis, dehydration and isomerization reactions (Sun BS. *et al.*, 2011)

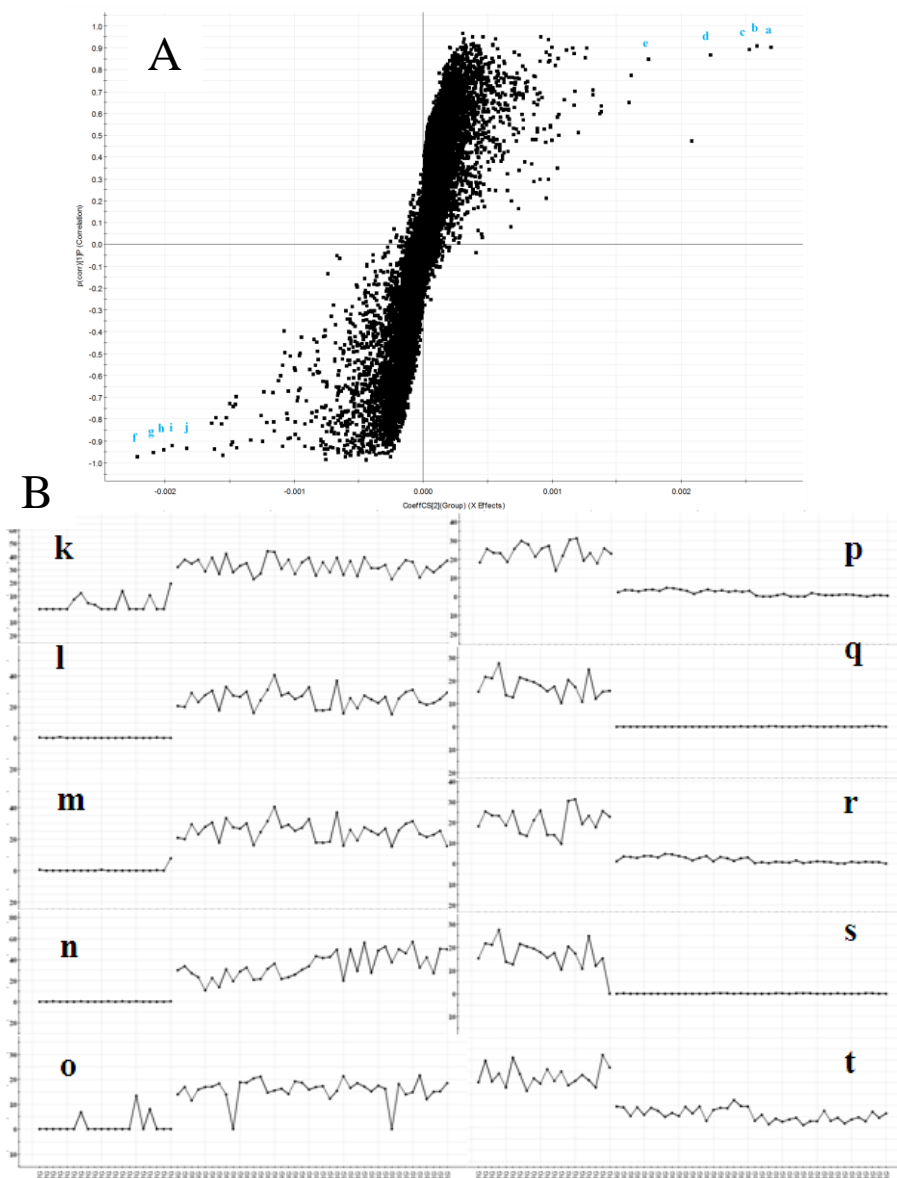


Fig.15 OPLS-DA/S-plots (A) and selected ion intensity trend plots (B) of fresh ginseng (FG), steamed ginseng (SG) and red ginseng (RG) samples from ESI⁺ mode data. **k** (t_R 0.79 min, m/z 268.1036), **l** (t_R 0.63 min, m/z 268.1036), **m** (t_R 0.65 min, m/z 268.1061), **n** (t_R 0.57 min, m/z 499.1985), **o** (t_R 16.60 min, m/z 425.3700), **p** (t_R 0.63 min, m/z 147.1029), **q** (t_R 29.18 min, m/z 425.2822), **r** (t_R 0.65 min, m/z 147.1068), **s** (t_R 29.20 min, m/z 425.2830), **t** (t_R 8.80 min, m/z 423.3549)

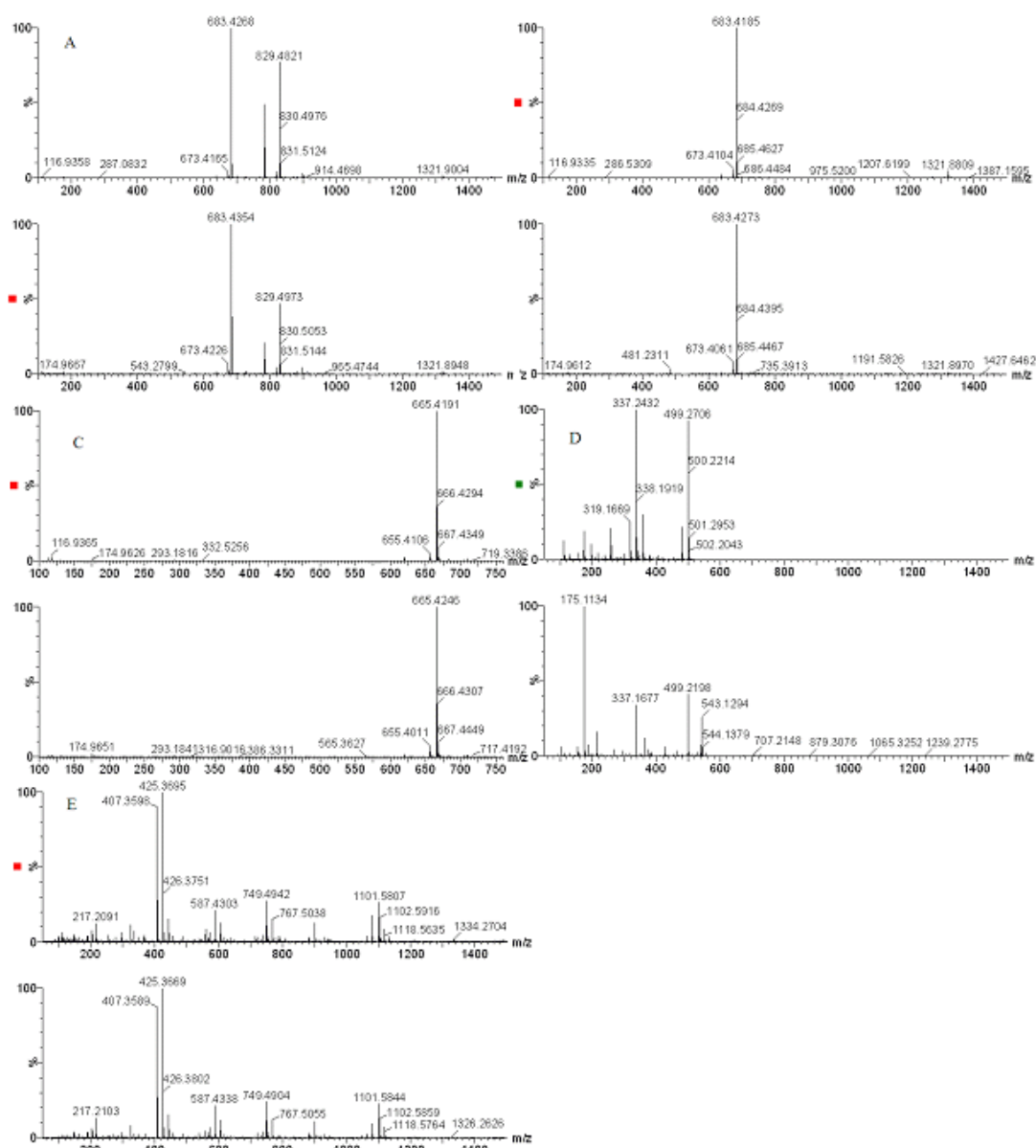


Fig.16 Spectra of reference standard (upper) and potential marker ions (lower). of identified ions ; (A) ginsenoside Rg2s and ion **a** (B) ginsenoside and ion **b** (C) ginsenoside Rh4 and ion **d** (D) Arginyl-fructose-glucose and ion **n** (E) ginsenoside Rc and ion **o**

3.2 Targeted analysis

To confirm nontargeted analysis, potential marker, arginyl-fructose was determined by using HPIC-PAD. The HPIC chromatograms of fresh ginseng, steamed ginseng and red ginseng are shown in Fig. 17. And the contents of the AFG generated in steaming process are represented in Table 8. AFG was found in SG and RG group but not found in FG group. The average contents of AFG in SG and RG group is 26.15 ± 6.99 and 65.90 ± 13.10 mg/g, respectively. These findings were in good agreement with previous nontargeted analysis result. Additionally, from this data, AFG was generated in more in drying step than steaming step.

Table.8 The contents of ginsenoside Rc fresh ginseng, steamed ginseng and red ginseng (n=20)

Analytes	Fresh ginseng (mg/g)	Steamed ginseng (mg/g)	Red ginseng (mg/g)
G-Rc	1.51 ± 0.69	1.54 ± 0.63	1.63 ± 0.72

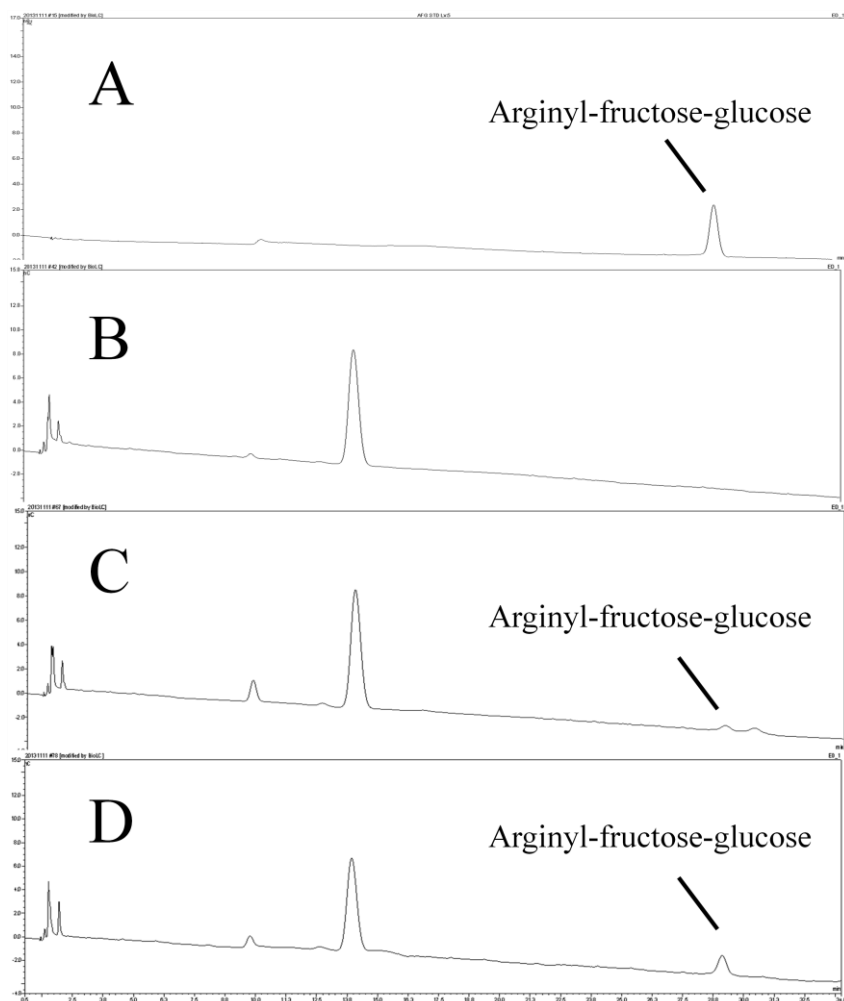


Fig.17 Representative HPIC chromatograms of arginyl-fructose-glucose (A), fresh ginseng (B), steamed ginseng (C) and red ginseng (D).

Table.9 The contents of arginyl-fructose-glucose in fresh ginseng, steamed ginseng and red ginseng (n=20)

Analytes	Fresh ginseng (mg/g)	Steamed ginseng (mg/g)	Red ginseng (mg/g)
AFG	N.D	26.15 ± 6.99	65.90 ± 13.10

N.D. : Not Detected

In non-targeted analysis, potential marker ion **e**, assumed quinquenoside R1, has a decarboxylated form of ginsenoside malonyl Rb1. It is known that malonyl group was hydrolyzed from malonyl ginsenoside during steaming process (Geoffrey C. Kite. *et al.*, 2003, Xie YY. *et al.*, 2012). Also, deacetylation was possible and it is need to analyze the contents of malonyl ginsenoside Rb1. Because there were no available commercial reference standards, malonyl-ginsenosides were analyzed by indirect base catalyzed-hydrolysis method as reported (William AC. *et al.*, 1996). The UPLC chromatograms of before and after base catalyzed-hydrolysis are shown in Fig. 18. It was examined that the height of peaks was increased after hydrolysis and the difference, indirect quantitated amount of malonyl ginsenoside, was calculated. And the contents of the malonyl ginsenosides were represented in Table 10.

It was confirmed that malonyl ginsenosides contents were high in fresh ginseng samples than in steamed ginseng and red ginseng. Ginsenoside malonyl Rb1 is most abundant malonyl ginsenoside in all groups. The average contents of ginsenoside malonyl Rb1 were 4.01, 2.08 and 1.75 mg/g at fresh, steamed and red ginseng, respectively. From these data, ginsenoside malonyl Rb1 was more decresed in steaming step (48.13 %) than drying step (15.96 %).

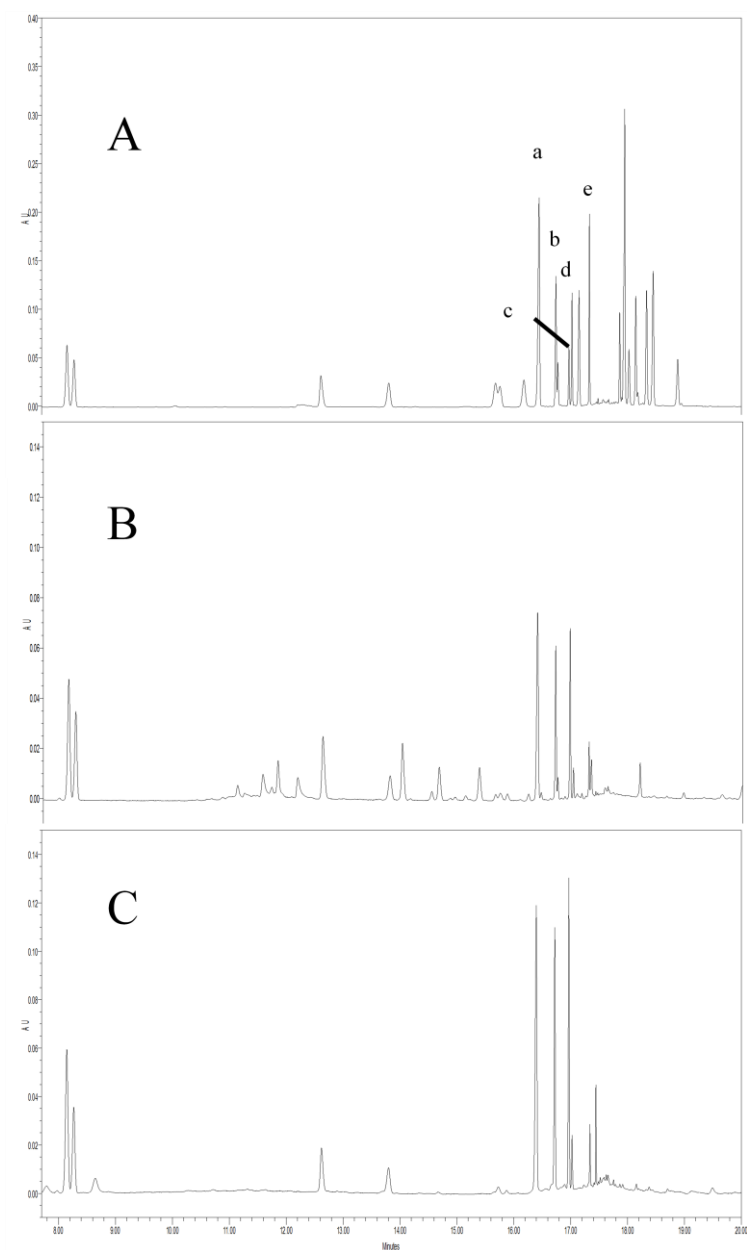


Fig.18 UPLC chromatograms of ginsenoside standards (A), and fresh ginseng sample before hydrolysis (B) and after hydrolysis (C). a : ginsenoside Rb1, b : ginsenoside Rc, c : ginsenoside Rb2, d : ginsenoside Rc, e : ginsenoside Rd

Table.10 The contents of malonyl ginsenosides in fresh ginseng, steamed ginseng and red ginseng (n=20)

	Ginsenoside	Ginsenoside	Ginsenoside	Ginsenoside	Ginsenoside
Analytes	malonyl Rb1	malonyl Rc	malonyl Rb2	malonyl Rb3	malonyl Rd
	(mg/g)	(mg/g)	(mg/g)	(mg/g)	(mg/g)
Fresh ginseng	4.01 ±2.17	1.23 ±0.53	1.98 ±0.84	0.31 ±0.11	0.21 ±0.17
Steamed ginseng	2.08 ±1.07	0.65 ±0.27	0.98 ±0.42	0.15 ±0.06	0.12 ±0.06
Red ginseng	1.75 ±0.81	0.52±0.39	0.82 ±0.39	0.11 ±0.05	0.09 ±0.04

IV. Comparative metabolomics for *Panax ginseng* of different cultivation period

1. Purpose of the present study

The ginseng root has been used as traditional herbal medicine for thousands of years in Asian countries, particularly in China, Korea and Japan. Ginseng is very slow-growing, usually grown for 4–6 years, perennial plants and six-years-old ginseng is most expensive because it has been known to have a better medicinal effect traditionally. So a reliable method to discriminate the cultivation age of *Panax ginseng* is required for quality control and prevention of its adulteration in the market (Yizhen Z. *et al.*, 2013, Kim NH *et al.*, 2011).

Because of recent developments in metabolomics and analytical instruments, we could detect major and minor metabolites. These nontargeted metabolomics helps to provide an understanding of the biochemical status of plants (Kim NH *et al.*, 2011). In previous research, the application of these metabolomic tools for discrimination of fresh or dried ginseng with different ages was tried by NMR (Yang SO. *et al.*, 2012, Shin YS. *et al.*), GC-TOF MS (Qiu Y. *et al.*, 2008) and UPLC-QTOF MS (Kim NH *et al.*, 2012).

In the present study, I performed metabolic fingerprinting fresh ginseng with different cultivation period (4 and 6 year) with GC-MS coupled with PCA. Also, UPLC-QTOF MS based metabolomic approach was performed to differentiate of processed ginseng (red ginseng) with different cultivation period (4 and 6 year).

2. Materials and methods

2.1 Materials

2.1.1 Ginseng samples

Four and six years old fresh ginseng (cultivated at Icheon, Gyeonggi-Do, by identical farmer, 10 roots) was processed to red ginseng by the Korea Ginseng Corporation (Daejeon, Korea). And four and six years old good grade red ginseng (same origin and farmer of fresh ginseng, 10 roots) were supplied by the Korea Ginseng Corporation. Samples were cut, freeze-dried, powdered and stored at -80°C

2.1.2 Chemicals and Reagents

Ginsenoside Rg1, Re, Rf, 20(S)-Rh1, Rb1, Rc, Rb2, Rd, 20(S)-Rg3 and 20(R)-Rg3 standards were purchased from Chromadex (Irvine, CA, USA) and ginsenoside Ro, 20(S)-Rg2, 20(R)-Rg2, 20(S)-Rh2, 20(R)-Rh2, F2, F4, Ra1, Rg6, Rh4, Rk3, Rg5, Rk1, Rb3, Rk2, Rh3 and notoginsenoside R1 standards were obtained from the Ambo Institute (Seoul, Korea). Formic acid (eluent additive for LC-MS) was purchased from Fluka (St. Louis, US). N, O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was purchased from SUPELCO (Bellefonte, PA, US). Phosphoric acid and sodium sulfate anhydrous was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). Methoxyamine hydrochloride, potassium *tert*-butoxide in THF and 3-hydroxymethylpyridine was purchased from Sigma-Aldrich Co (St. Louis, MO, US). HPLC-grade acetonitrile, hexane, chloroform and methanol were purchased

from Merck (Darmstadt, Germany). All distilled water used in this experiment was purified by the Milli-Q gradient system (Millipore, Bedford, MA, USA) and the resistance value was measured as 18 M Ω prior to use.

2.2 Methods

2.1.1 Sample preaparation for GC-MS

The sample preparation of red ginseng powders and concentrates were performed as similar manner as previous reports (Jan L. *et al.*, 2006). Freeze-dried powder (0.1 g) was weighed and 1.4 mL of extraction solvent (methanol:H₂O = 8:2) was added. The powder was then shaken for 10 min at 60 °C in a thermomixer (DeepWellMaximizer/BioShaker M.BR-022UP, TAITEC, Saitama-ken, Japan) at 950 r.p.m. After centrifugation (MICRO-12, Hanil Science Industrial Co. Inchun, Korea) for 10 min at 10770 g, supernatant was trasfered to a Schott GL14 glasss vial. For liquid-liquid partition chloroform (0.75 mL) and distilled water (1.5 mL) added and centrifugation was performed for 15 min at 5162g. To concentrate, the lower phase (non-polar phase, 0.3 mL) was trasfered into a fresh 1.5 mL tube and dried using vacuum concentrator concentrator (Modulspin 40, Hanil Science, Inchun, Korea) without heating. For derivatization, 0.04 mL of methoxyamine hydrochloride solution (20 mg/mL in pure pyridine) was added and shaken for two hours at 37 °C. And then 0.07 mL of BSTFA (containing 1% TMCS) reagent was added and shaken for 30 min at 60 °C. Prepared sample was injected into the GC-MS system.

2.1.2 Sample preparation for identifying dehydrocrepenate

Picolinyl ester of dehydrocrepenate for GC-MS analysis was prepared from extract of chanteterelle mushrooms purchased at a local grocery store. The mushrooms were initially pulverized to a fine powder with a mortar and pestle. The powder (10 g) was homogenized with methanol (100 mL) for 1 min. After homogenization, Sonication was performed with chloroform/methanol mixture (vol/vol=2/1, 500 mL) for one hour. The extract was dried over anhydrous sodium sulphate and evaporated. The dried sample was dissolved in dry dichloromethane (1 mL). A solution of potassium *tert*-butoxide in THF (0.1 mL, 1.0 M) was added to 3-hydroxymethylpyridine (0.2 mL) to form the reagent. The reagent was added to sample solution and the mixture was held at 40 °C for 30 min in a closed vial. After cooling at room temperature, water (5 mL) and hexane (10 mL) were added. The unit was then vortexed and the organic phase was collected, dried over anhydrous sodium sulphate and evaporated. The sample was finally dissolved in hexane (1 mL) for GC-MS analysis.

In the case of ginseng sample, the concentrate of nonpolar phase in VI-2.2.1 was used for preparation of picolinyl ester. It dissolved in dry dichloromethane (1 mL) and sample preparation was carried out according to VI-2.2.2 with these solutions.

2.2.3 GC-MS condition for analyzing non-polar metabolites

Analysis of the derivatives was performed by GC-MS QP2010 (SHIMADZU Co. Kyoto, JAPAN) with capillary column of DB-5MS (60 m X 0.25mm i.d, 0.25 μ m film thickness, Agilent Technologies, Santa Clara, CA, US). GC-MS operated

in the electron impact mode at 70 eV. The temperatures of injector and interface were maintained at 280 °C. Separation was achieved with an oven temperature linearly increasing from 80 (2 min constant temperature) to 280 °C (31 min constant temperature) at 7.0 °C/min. Helium was employed as the carrier gas at a constant flow rate of 1.0 mL/min.

2.2.4 GC-MS condition for identifying dehydrocrepenate

GC-MS condition for identifying dehydrocrepenate was similar to previous non-polar metabolites analysis condition. QP2010 GC-MS with capillary column of DB-5MS was used for analysis. GC-MS operated in the electron impact mode at 70 eV. The temperatures of injector and interface were maintained at 285 and 280 °C, respectively. Separation was achieved with an oven temperature linearly increasing from 230 (25 min constant temperature) to 295 °C at 1.5 °C/min. Helium was employed as the carrier gas at a constant flow rate of 1.0 mL/min.

2.2.5 Sample preparation for UPLC-QTOF MS

The sample preparation was performed in a similar manner to II-2.2.1. A half gram of the freeze-dried and grounded processed ginseng sample was weighed in a centrifugal tube (15 mL, PP-single use; BioLogix Group, Jinan, Shandong, China) and shaken vigorously after the addition of 10 mL of 70% methanol. Then the extraction was placed in an ultrasonic cleaner (60 Hz; Wisclean, Seoul, Korea) for 30 min. The solution was centrifuged (Legand Mach 1.6R; Thermo, Frankfurt, Germany) at a speed of 3000 rpm/min for 10 min and the aliquot of the supernatant solution was filtered (0.2 µm; Acrodisc, Gelman Sciences, Ann Arbor, MI, USA).

100 μ L of filtered sample was diluted tenfold with 70 % methanol and injected into the UPLC-QTOF MS system (Waters Co., Milford, MA, USA).

2.2.6 UPLC-QTOF MS data processing and statistical analyses

Raw GC-MS data of non-polar fractions were normalized by dividing the peak area of specific metabolites in each sample with average peak area. Principal component analyses of processed data were performed with STATISTICA (StatSoft Inc., ver. 7.0). Correlations between the concentrations of individual metabolites were also evaluated with the same software, while the heatmap of correlation matrix were constructed with Heatmap Builder ver. 1.1 (King JY. *et al.*, 2005).

To evaluate the potential characteristic age dependent-components of red ginseng, the ESI⁻raw data of all samples was calculated with MassLynxTM application manager version 4.1 (Waters MS Technologies, Manchester, UK). The method parameters were same as II-2.2.4. For data analysis, a list of the intensities of the detected peaks was generated using the pair of retention time (t_R) and mass data (m/z) as the identifier of each peak. A tentative ID was assigned to each of these t_R - m/z pairs for data adjustment that was based on their chromatographic elution order of UPLC. Ions from different samples were considered to be the same when they showed the identical t_R and m/z values. MarkerLynxTM (Waters MS Technologies, Manchester, UK) was used for the normalization of each detected peak against the sum of the peak intensities within that sample. The resulting data consisted of a peak number (t_R - m/z pair), sample name and ion intensity. Then the consequent data sets were analyzed by principal component analysis (PCA) and orthogonal partial least squared discriminant analysis (OPLS-DA) using the MarkerLynxTM.

2.2.7 UPLC-QTOF MS condition for analyzing metabolites

The instrumental analysis was performed by Ultra Performance Liquid Chromatography (UPLC) using an ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 µm; Waters Co., Milford, MA, USA) on Waters ACQUITY UPLC system with a binary solvent manager, sample manager and photo diode array detector. The column temperature was 40°C. The binary gradient elution system consisted of 0.01% formic acid in water (A) and 0.01% formic acid in acetonitrile (B). The separation was achieved using the following protocol: 0-0.5 min (15% B), 14.5 min (30% B), 15.5 min (32% B), 18.5 min (38% B), 24.0 min (43% B), 27.0 min (55% B), 27.0 -31.0 min (55% B), 35.0 min (70% B), 38.0 min (90% B), 38.1 min (15% B), 38.1-43.0 min (15% B). The flow rate was set 0.4 mL/min and the sample injection volume was 2.0 µL.

Metabolite profiling of red ginseng sample was performed by coupling a Waters ACQUITY UPLC system to a Waters Xevo Q-TOF mass spectrometer (Waters MS Technologies, Manchester, UK) with electrospray ionization (ESI) interface. The source and desolvation gas temperatures were kept at 400 and 120°C, respectively. The nebulizer and desolvation gas used was N₂ gas. The flow rates of the nebulizer gas and cone gas were set at 800 and 50 L/h, respectively. The capillary and cone voltages were adjusted to 2300 and 40 V, separately. The mass accuracy and reproducibility were maintained by infusing lockmass (leucine-enkephalin, 200 pg/L) thorough LocksprayTM at a flow rate of 7 µL/min. Centroided data was collected for each sample from 100 to 1450 Da and the *m/z* values of all acquired spectra were automatically adjusted during acquisition based on lockmass and dynamic range enhancement. The accurate mass and molecular formula assignments were obtained with MassLynxTM 4.1 software

(Waters MS Technologies, Manchester, UK).

2.2.8 Malonyl ginsenosides analysis

Ginsenoside Rb1 standards were purchased from Chromadex (Irvine, CA, USA)

The ginseng sample preparation was performed in a similar manner as III-2-2.1. And malonyl-ginsenosides Rb1 were analyzed by indirect base-hydrolysis method as reported (Court WA. *et al.*, 1996). A half gram of ginseng powder was weighed in a centrifugal tube (15 mL, PP-single use; BioLogix Group, Jinan, Shandong, China) and shaken vigorously after the addition of 10 mL of 70% MeOH. Extraction was performed in an ultrasonic cleaner (60 Hz, Wiseclean, Seoul, Korea) for 30 min. After ultrasonic extraction, centrifugal separation (Legand Mach 1.6R; Thermo, Frankfurt, Germany) was performed for 10 min at 3000 rpm. The resulting supernatant solution was filtered (0.2 μ m; Acrodisk, Gelman Sciences, Ann Arbor, MI, USA) and injected into the Ultra Performance Liquid Chromatography (UPLC) system (Waters Co., Milford, MA, USA). And acidic ginsenosides were hydrolysed by adding 80 μ L of 5% KOH to a portion (850 μ L) of the ginseng extract. After 2 h the solution was neutralized by adding 80 μ L of a 0.01 M KH_2PO_4 solution. The mixture was diluted with 850 μ L of acetonitrile and analyzed to determine total ginsenosides.

The instrumental conditions of UPLC as follows. The chromatographic separation obtained by using an ACQUITY BEH C18 column (100 mm \times 2.1 mm, 1.7 μ m; Waters Co., Milford, MA, USA) and the column temperature was 40°C. The binary gradient elution system consisted of 0.01 M KH_2PO_4 in water (A) and acetonitrile (B). The separation was achieved using the following protocol; 0-0.5

min (15% B), 14.5 min (30% B), 15.5 min (32% B), 16.5 min (40% B), 17.0 min (55% B), 21.0 min (90% B), 25.0 -27.0 min (15% B). The flow rate was set 0.6 mL/min and the sample injection volume was 2.0 μ L. The ginsenosides were determined at a UV wavelength of 203 nm using a Photo Diode Array detector (PDA).

3. Results and Discussion

3.1 GC-MS analyses of non-polar metabolites of fresh ginseng

Fresh ginseng extracts for non-polar fraction were analyzed with GC-MS to characterize the chemical identities of metabolites. Among 58 peaks observed in GC-MS, 38 metabolites were identified as TMS-derivatives through mass spectral library search while 20 were not able to be characterized properly. Major constituents in non-polar fraction were polyacetylenes (30%), free fatty acids (38%), and monoacyl glycerols (17%). Short chain organic acids, terpenes, and plant steroid comprised approximately 3% of total metabolites (Table 11).

Numerous numbers of different polyacetylenes have been reported in Apiaceae and Araliaceae plants (Christensen LP *et al.*, 2008, Hansen *et al.*, 1986, Lee *et al.*, 2009, Zidorn *et al.*, 2005). Panaxynol (also called as falcarinol) and panaxydol were known as major polyacetylenes in ginseng root (Liu *et al.*, 2007, Nho *et al.*, 1989). Two peaks at retention time of 29.859 and 31.864 min were identified as panaxynol and panaxydol (Table 11 and Fig. 19 and 20), occupying 5-7 and 22-26% of total metabolites, respectively.

Table.11 Profiles of polyacetylenes and selected lipids, involved in polyacetylene biosynthesis in *Panax ginseng* root

Class	Name	Peak ID ^a	Rt (min) ^b	MW	Cultivation period				Ratio (6/4) ^d
					4 year		6 years		
					Average ^c	CV	Average	CV	
Polyacetylene	Panaxynol	1	29.859	316	6924049	97.1	15508163	57.9	2.2
	Panaxydol	2	31.864	332	32832396	67.7	47202248	47.7	1.4
Free fatty acid	C18:2	3	31.704	352	17896667	78.2	27173948	34.9	1.5
	C18:1	4	31.756	354	5130026	67.7	7548302	45.1	1.5
	C18:0	5	32.092	356	1683237	47.4	2572375	25.5	1.5
	Dehydrocrepe- nynic acid (DHC)	6	32.438	348	714227	83.7	1326676	44.4	1.8
Monoacyl- glycerol	MGC18:2	7	41.196	498	301455	56.9	519609	61.8	1.7
	MGC18:1	8	41.936	500	986156	39.2	1334817	27.5	1.4
	MGC18:0	9	43.064	502	8617903	33.9	14538780	17.2	1.7

^aPeak ID in figure. 19^bAbbreviations: Rt, Retention time (min); MW, molecular weights of TMS derivatives; CV, coefficient of variation.^cAverage GC-MS peak areas of 10 samples^dRatio of average peak areas of selected metabolites between 6-year and 4-year samples.

Table.12 Non-polar metabolite profiles of *Panax ginseng* root, with different cultivation period (6 years / 4 years)

Class	Name of metabolite	Rt (min)	MW	Peak area						Ratio (6/4)
				6 years			4 years			
				Average ^a	SD	% Total ^b	Average	SD	% Total	
Polyacetylene	Panaxynol	29.859	316	15508163	8985247	7.54	6924049	6723544	5.41	2.24
	Panaxydol	31.864	332	47202248	22519162	22.95	32832396	22223729	25.67	1.44
Fatty acid	FA C6:0 (?)	10.537	188	185854	57559	0.09	60047	23006	0.05	3.10
	FA C6:0	10.824	188	442455	256500	0.22	167990	96840	0.13	2.63
	FA C7:0	12.994	202	181704	61216	0.09	46117	31271	0.04	3.94
	FA C9:0	17.250	230	83933	39067	0.04	30039	31081	0.02	2.79
	FA C12:0	22.917	272	109856	70278	0.05	16767	23968	0.01	6.55
	Undecenoic/Undecunoic acid	24.309	256/254	1177810	441901	0.57	169682	99737	0.13	6.94
	Azelaic acid	25.333	332	602844	273459	0.29	100716	44119	0.08	5.99
	FA C14:0	26.277	300	368181	88138	0.18	155656	137471	0.12	2.37
	FA C15:0	27.783	314	925469	230700	0.45	286002	405372	0.22	3.24
	FA C16:1	28.940	326	1835438	634881	0.89	448265	462475	0.35	4.09
	FA C16:0	29.258	328	26013581	5868134	12.65	15012368	9001467	11.74	1.73
	FA C17:0	30.668	342	564309	182186	0.27	533217	322856	0.42	1.06
	FA C18:2	31.704	352	28173948	9097366	13.70	17896667	13996485	13.99	1.57
	FA C18:1, 1	31.756	354	7548302	3405663	3.67	5130026	3473788	4.01	1.47
	FA C18:1,2	31.864	354	4396550	2493527	2.14	3283239	2222373	2.57	1.34
	FA C18:0	32.092	356	2572375	655542	1.25	1683237	797972	1.32	1.53
	Dehydrocrepenynic acid	32.438	348	1026676	455700	0.50	714227	597745	0.56	1.44
	C18:1 with 2 triple bond (?)	34.116	344	700127	237876	0.34	806897	744108	0.63	0.87
	FA C20:0	35.238	384	220544	83492	0.11	173419	85108	0.14	1.27
	FA C21:0	37.083	398	167576	120669	0.08	109018	141282	0.09	1.54
	FA C22:0	39.325	412	532393	237609	0.26	243599	245945	0.19	2.19
Acylglycerol	MAG-C8:0	25.793	362	118887	55899	0.06	92397	36220	0.07	1.29
	MAG-C16:0, 1	37.171	474	1373418	270801	0.67	873996	267704	0.68	1.57
	MAG-C16:0, 2	37.917	474	12003656	1683607	5.84	6526618	2642922	5.10	1.84
	MAG-C17:0	40.254	488	418281	140795	0.20	173542	96364	0.14	2.41
	MAG-C18:2	41.196	498	519609	320889	0.25	301455	171545	0.24	1.72
	MAG-C18:1, 1	41.936	500	1334817	367554	0.65	986156	386218	0.77	1.35
	MAG-C18:1, 2	42.273	498	4358844	1691502	2.12	2102683	1243470	1.64	2.07
	MAG-C18:0	43.064	502	14538780	2504847	7.07	8617903	2924004	6.74	1.69
	MAG-C20:0	50.819	530	366628	170249	0.18	58869	45017	0.05	6.23

(continued)

Class	Name of metabolite	Rt (min)	MW	Peak area						Ratio (6/4)
				6 years			4 years			
				Average ^a	SD	% Total ^b	Average	SD	% Total	
Misc	Lactic acid	10.422	234	571790	157404	0.28	154786	72995	0.12	3.69
	Fumaric acid	20.091		203723	131257	0.10	12355	13123	0.01	16.49
	Terpene 1	23.644		3106748	1370639	1.51	3919460	1857820	3.06	0.79
	α-Glycerophosphate	24.552	460	216121	180590	0.11	47918	15482	0.04	4.51
	Vanillic acid	24.779	312	89176	62859	0.04	16873	8566	0.01	5.29
	Alkanol	31.372		817687	322907	0.40	425470	258606	0.33	1.92
Steroid	Stigmasterol	71.367	484	896541	346527	0.44	393411	302416	0.31	2.28
Unknown	Unknown 1	29.130	328	339698	147405	0.17	20280	37910	0.02	16.75
Unknown	Unknown 2	29.602	318	184991	121057	0.09	103961	96070	0.08	1.78
	Unknown 3	30.441		303754	105121	0.15	132400	79698	0.10	2.29
	Unknown 4	30.592		613144	288180	0.30	464263	397112	0.36	1.32
	Unknown 5	31.019		138913	70526	0.07	187218	216550	0.15	0.74
	Unknown 6	31.203		296699	186758	0.14	268847	127085	0.21	1.10
	Unknown 7	31.500		1040589	651531	0.51	5384754	2332518	4.21	0.19
	Unknown 8	31.601		3410482	1537039	1.66	2876756	1381736	2.25	1.19
	Unknown 9	32.718		1272610	440984	0.62	575317	280079	0.45	2.21
	Unknown 10	32.847		788081	321338	0.38	335331	191249	0.26	2.35
	Unknown 11	33.593		5954876	2351924	2.90	2363020	1525287	1.85	2.52
	Unknown 12	33.732		984493	876446	0.48	357053	153620	0.28	2.76
	Unknown 13	34.328		2725401	977514	1.33	2142800	1145882	1.68	1.27
	Unknown 14	34.589		1579512	810635	0.77	393124	159263	0.31	4.02
	Unknown 15	34.848		2006766	1600649	0.98	639799	452281	0.50	3.14
	Unknown 16	36.350		890099	368421	0.43	310347	185004	0.24	2.87
	Unknown 17	37.375		379141	358689	0.18	140144	139586	0.11	2.71
	Unknown 18	37.470		642366	216031	0.31	61851	51886	0.05	10.39
	Unknown 19	43.708		259872	210798	0.13	73218	67023	0.06	3.55
	Unknown 20	51.098		347810	60064	0.17	287787	103238	0.23	1.21

^aData from 10 different samples from each cultivation period (total 20 samples)

^b% of each component

Abbreviations: Rt, retention time in min; MW, molecular weights of TMS derivatives; SD, standard deviation; Min, minimum peak area; Max, maximum peak area

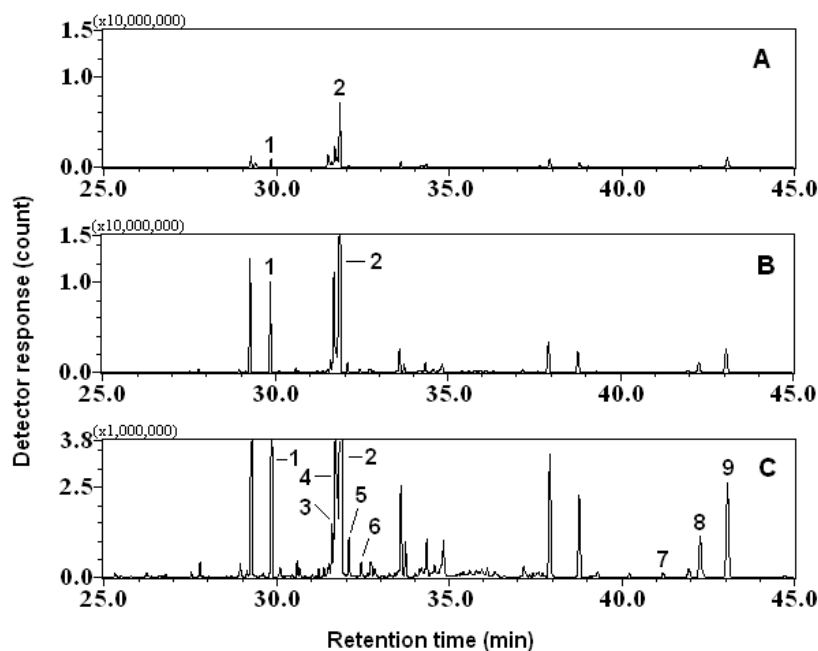


Fig.19 Representative GC-MS total ion chromatograms of non-polar metabolites in *Panax ginseng* root, cultivated for 4 (A), 6 years (B), and signal-enhanced chromatogram of insert B (C). Peak numbers in chromatograms denote the metabolites in table 1.

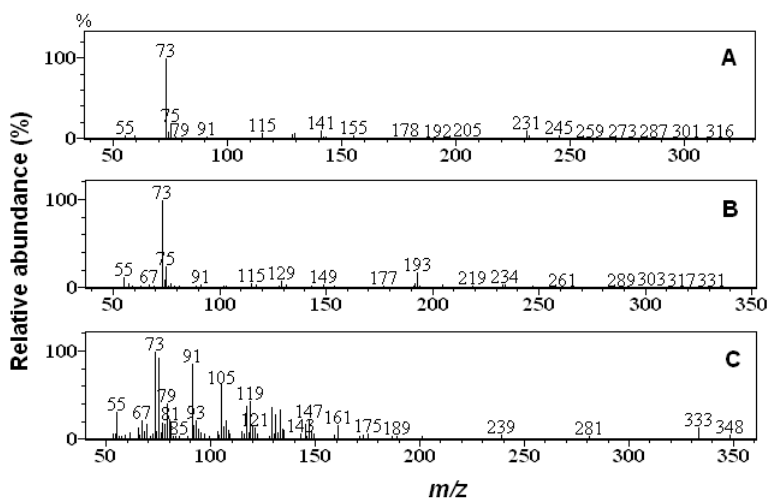


Fig.20 Mass spectra of TMS-derivatized panaxynol (A), panaxydol (B), and dehydrocrepenynic acid (C)

Approximately 21 free fatty acids were detected, among which the fatty acids with 6 to 22 carbons were observed with different concentrations. Palmitic acid (16:0) and linoleic acid (C18:2) were the most abundant fatty acids, comprising 12.7 and 13.7% of total metabolites, respectively. Relative concentration of unsaturated fatty acids, including palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and some metabolites with triple bond were approximately 21.8%, while those of saturated fatty acids were 16.9%.

Particularly, one (t_R , 32.438 min) was tentatively identified as dehydrocrepenynic acid (DHC) (6, Fig. 19C and Figure 20C), even though octadecadiynoic acid and dehydrocrepenynic acid have the same molecular weight, because DHC is a well-known intermediate of panaxynol biosynthesis (Hansen *et al.*, 1986). In addition, DHC was identified by comparison of mass spectrum of ginseng and chanterelle mushrooms pycolynil ester derivatives (Fig. 22). It was known that DHC typically composes of > 25 % of the total fatty acids of chanterelle mushrooms (Lizhi Z. *et al.*, 2001). In addition to known fatty acids, some peaks showed characteristic mass spectra of unsaturated fatty acids (t_R , 24.309 and 34.116 min).

In addition to free fatty acids, several monoacylglycerols (MAGs) were also observed (approximately 17% of total metabolites). Acyl groups of MAGs were comprised of fatty acids with eight to twenty carbons. In comparison with free fatty acids, MAGs with saturated fatty acyl groups were dominant (Table 12). Several metabolites, other than fatty acids or MAGs were also identified, including lactic acid, fumaric acid, and precursors of other metabolic pathways. Among plant steroids, stigmasterol was comprised with 0.5% of total metabolites, while other steroid was found in trace quantities. Many terpenes

were characterized from the volatile oils of ginseng root (Qiu *et al.*, 2008 and Richter *et al.*, 2005). Spathulenol (t_R , 23.644 min) was observed in all samples (approximately 0.79-1.51% of total metabolites), while other terpenoid metabolites were found in trace quantities.

Many metabolites had no reasonable matching compounds from spectral library search. However, most of their mass spectra were highly similar with those of long-chain fatty acids or alcohols.

In general, concentrations of non-polar metabolites, judged from peak area in GC-MS analysis indicate that 6-year root contains higher amount of metabolites than those of 4 years samples (Table 11).

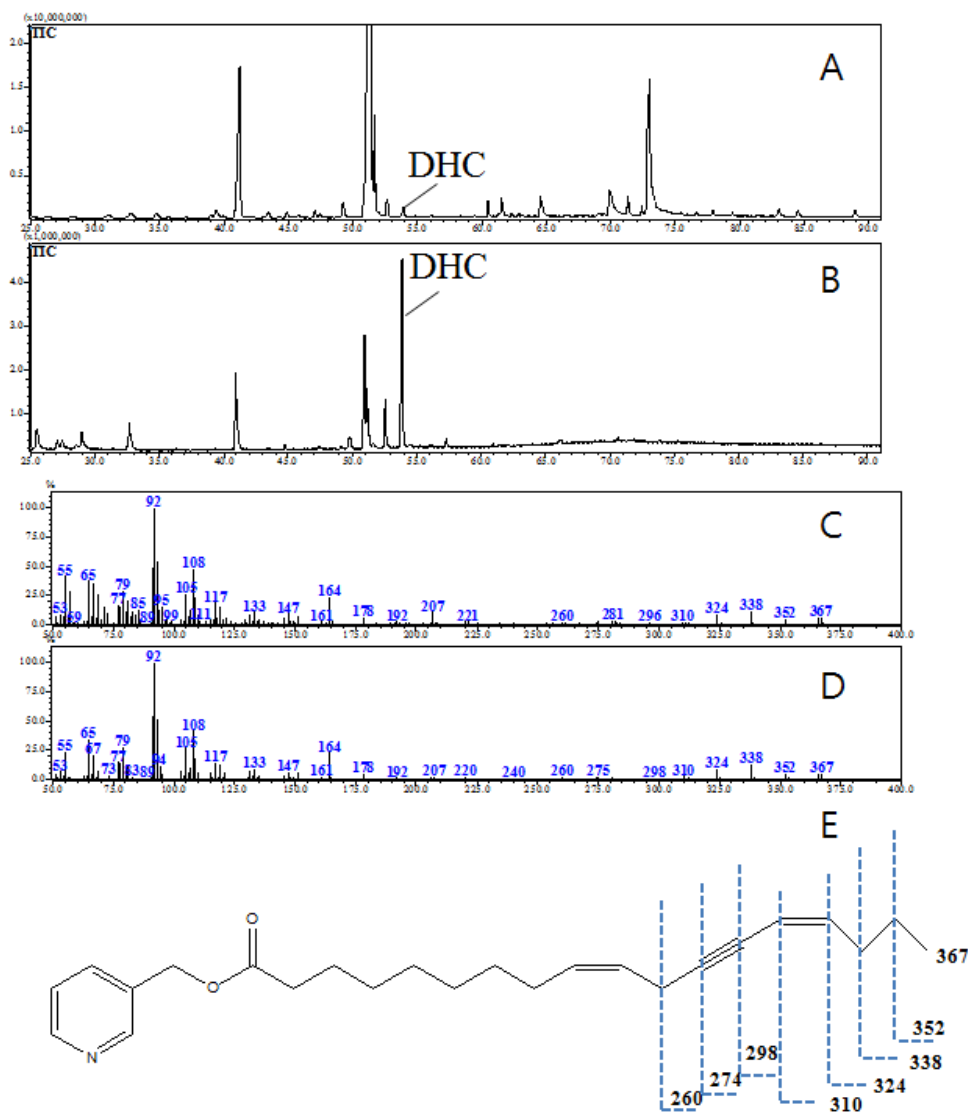


Fig.21 Total ion chromatograms (A: ginseng, B: chanterelle mushroom), mass spectra (C: ginseng, D: chanterelle mushroom) and proposed fragmentation pattern of DHC pycolynil ester (E).

3.2 Statistical analyses of non-polar metabolite profiles

PCA of non-polar metabolites, based on GC-MS data has been performed. Samples of different cultivation period (4 and 6 years) were clearly separated each other (Fig. 22). Approximately 60% variance can be explained with three principal components. Samples of 4 year root were more tightly clustered together than those of 6-years. PCA analysis clearly indicates that non-polar metabolite profiles of ginseng root tissues changes with cultivation periods. It is commonly accepted that bioactive secondary metabolites profiles in ginseng root (e.g., ginsenosides) are rapidly change during cultivation period. In general, rapid accumulation of these metabolites is observed after 5-6 years while the level is kept low till 3-4 years (Yamaguchi *et al.*, 1988). Recently, Qiu *et al.* (2008) has proven that composition of volatiles from root also give clear separation, depending on root ages. Yamakuchi *et al.* proposed that the rapid growth of root periderm/phloem after 4 years may be related to the accumulation of some bioactive metabolites (Yamaguchi *et al.*, 1988). *In situ* analysis with Raman spectroscopy indicates that polyacetylenes (panaxynol) are strongly accumulated in phloem tissues of carrot root (Baranska *et al.*, 2005). In consideration of these reports, age-dependent changes of root growth may cause the metabolic differentiation.

In addition, this study and previous research suggested that both polar and non-polar metabolite profiles are rapidly changed during the cultivation period and both class of metabolites can be used to discriminate the ginseng root of different ages.

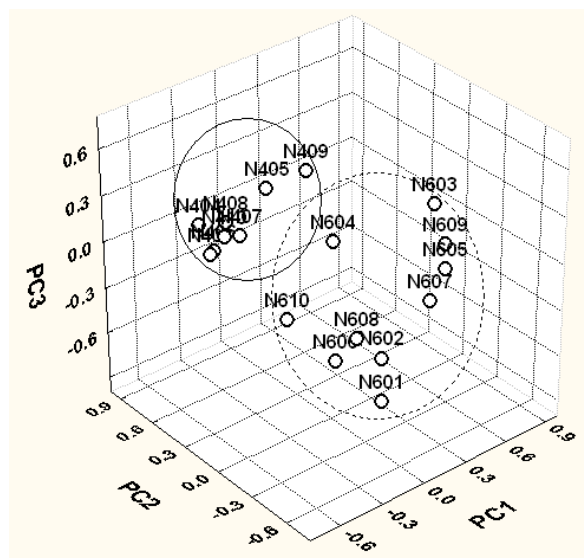


Fig.22 Principal component analysis of non-polar metabolites from *Panax ginseng* root, cultivated for 4 and 6 years with GC-MS.

3.3 Biosynthesis of panaxynol and panaxydol

Polyacetylenes from Araliaceae usually contains 17 carbons with one or two triple bonds (Hansen *et al.*, 1986). Oleic or linoleic acids were considered to be biosynthetic precursors (Fig.23). More recently, Minto *et al.* (2008) gave detailed reviews of the polyacetylene biosynthesis from various organisms (Minto *et al.*, 2008). Relationship between fatty acids and polyacetylenes with 17 carbon units was again proposed in this review. These biosynthetic pathways suggested that similar correlations may also be found in GC-MS results of

polyacetylenes and its possible precursors.

To identify the relationships, correlation matrices were constructed (Fig. 24). The results suggested that there are strong correlations between polyacetylenes and other metabolites, including oleic acid, linoleic acid, DHC, and monoacyl glycerol (ester of C18:1 acid in unknown position of glycerol). Examples of these analyses were presented in Fig. 25. Strong correlation between panaxynol and panaxydol (correlation coefficient $R^2 = 0.8467$) suggested that the metabolism of these metabolites are closely inter-connected. Although no details are available for the transformation of panaxynol to panaxydol, it is reasonable to postulate that oxidative enzymes (e.g., cytochrome P450s) may be involved in this step. The levels of polyunsaturated fatty acid (e.g., linoleic acid) also have shown a linear correlation with those of polyacetylenes while no clear relationships were observed with saturated or mono-enoic fatty acids (Fig. 25). The concentration of DHC also shows a linear correlation with polyacetylenes (Fig. 25). In addition, similar relationship was found between the level of DHC and linoleic acid (Fig. 24)

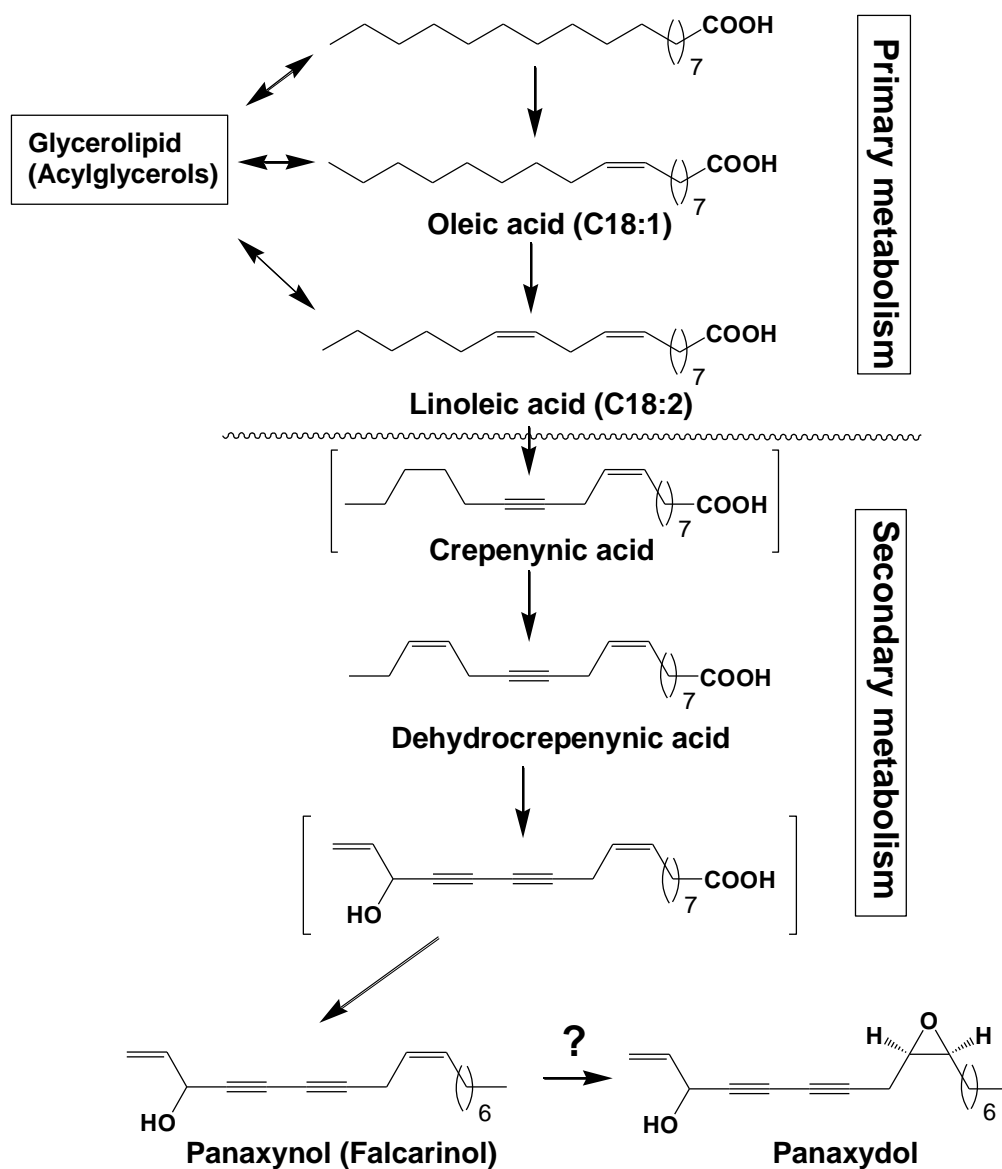


Fig.23 Biosynthetic pathway of panaxynol (falcarinol) and panaxydol. Metabolites in bracket were not found in this study.



Fig.24 Correlation matrix of non-polar metabolites

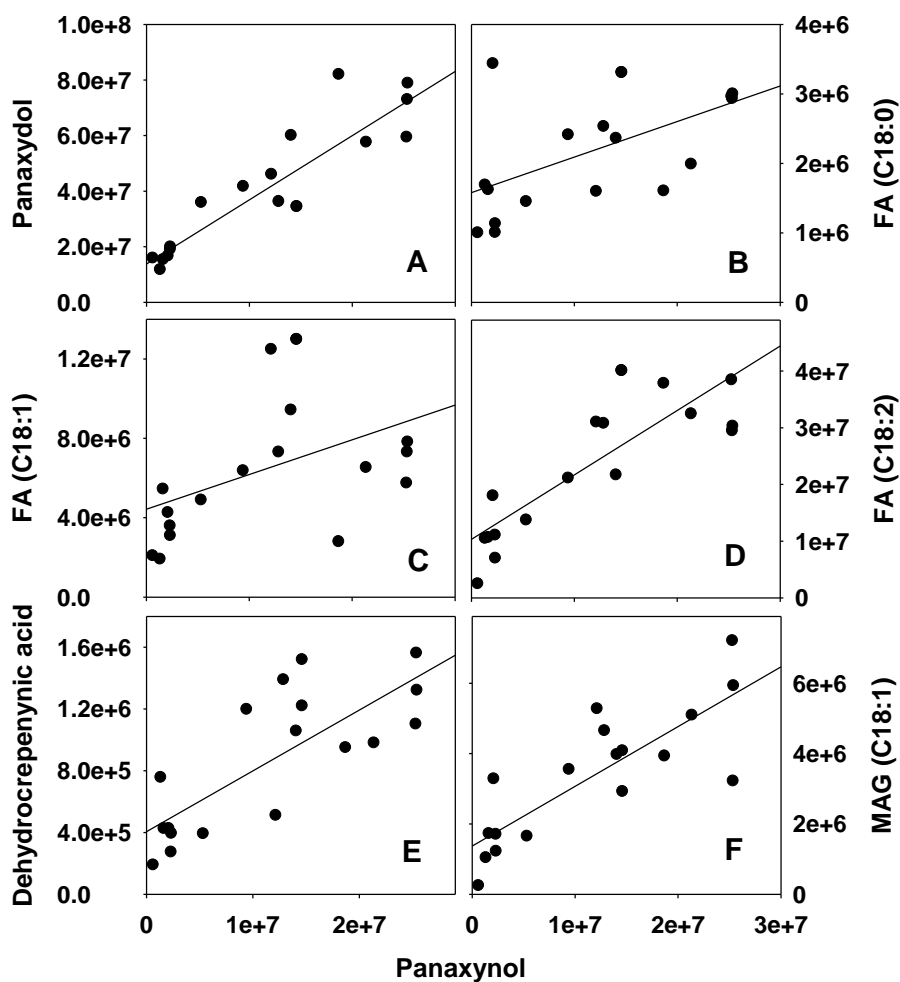


Fig.25 Linear correlation between the GC-MS peak areas of panaxynol and other metabolites, including panaxydol (**A**), octadecanoic acid (**B**), *cis*-9-octadecenoic acid (**C**), *cis,cis*-9,12-octadecadienoic acid (**D**), dehydrocrepenynic acid (**E**), and 1-(9,12-octadecadienoyl)-glycerol (**F**).

The results supported that linoleic acid is one of the most important precursor of polyacetylene biosynthesis in ginseng. Statistical analyses with monoacyl glycerols give interesting differences with those of free fatty acids. For example, the level of free mono-enoic acid did not show clear correlation with polyacetylenes, while the concentration of oleoyl-glycerol was linearly increased with panaxynol (Fig. 25C and F). It has to be mentioned that monoacyl glycerol can be a reservoir of fatty acids or precursors of storage lipids in plant. According to correlation analyses, it was evident that there are strong interactions between polyacetylenes and some monoacyl glycerols (e.g., MGC18:1). It is not clear why glycerol ester of oleic acid (MGC18:1), rather than linoleic acid gives strong correlation with polyacetylenes. However, it can be suggested that MGC18:1 may be a precursor of free oleic acid, which is transformed into linoleic acid and precursors of polyacetylenes.

It is noteworthy that some uncharacterized metabolites (**UK11** and **13**, t_R , 33.593 and 34.328 min) also have shown strong correlation with polyacetylenes (Table 12, Fig. 24). **UK13** gives reasonable relationships only with polyacetylenes while many fatty acids and monoacyl glycerols have shown positive correlations with **UK11**. Further researcher with purified metabolites will give the identities of these metabolites.

3.4 Nontargeted UPLC-QTOF MS analysis of processed ginseng sample with different cultivation period

UPLC-QTOF MS data was used for the non-targeted component analysis for searching potential marker of precessed ginseng (red ginseng) with different cultivation period (4 and 6 year). The chromatograms of each red ginseng group, namely 4 year group and 6 year group (4Y and 6Y, respectively), were generated with the analysis time of 43 min as II-3.2 nontargeted analysis. The gradient elution mode was employed in the UPLC system to acquire the maximized chromatographic performance including simultaneous data acquisition and appropriate retention times and integration values. Fig. 26 shows the total ion chromatograms (TIC) of 4Y and 6Y. The accurate mass measurement was established by the simultaneous but independent acquisition of reference ions of leucine-enkephalin (m/z 554.2615) via the LockSprayTM interface. This system offered several advantages for non-targeted metabolite profiling, including minimization of prevention of fluctuations in reference ionization efficiency according to the gradient elution.

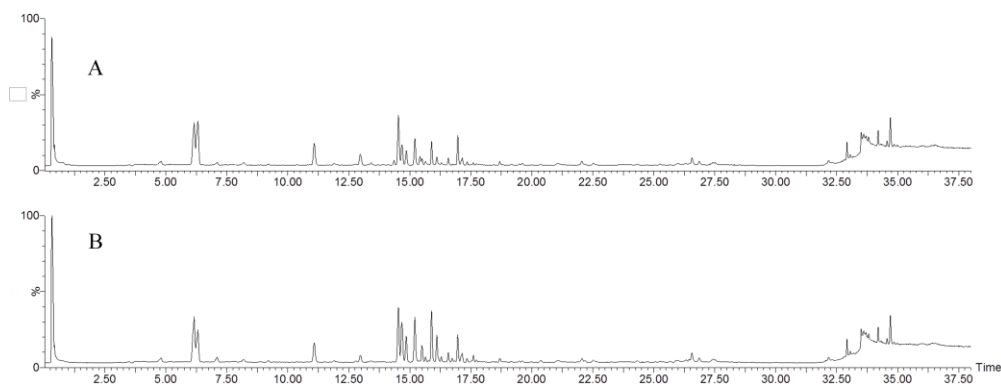


Fig.26 Representative total ion chromatograms of processed ginseng samples. (A) 4Y group (B) 6Y group

In order to find novel discrimination marker ions for 4Y and 6Y an unsupervised PCA and supervised OPLS-DA were performed using the UPLC-QTOF MS data. After creating a process, I could get the results table, displaying the ID, mass and retention time of the marker. Using Ez info software, built in Markerynx, I performed PCA analysis and data was displayed as score plots (Fig. 27). As shown in Fig. 26, 4Y and 6Y samples were clearly clustered into two groups. This means that the holistic qualities of 4Y and 6Y were consistent with each other and indeed different in the levels or occurrences of their components.

To explore the potential chemical markers that contributed most to the differences between the groups, UPLC-QTOF MS data from these samples was processed by a supervised OPLS-DA.

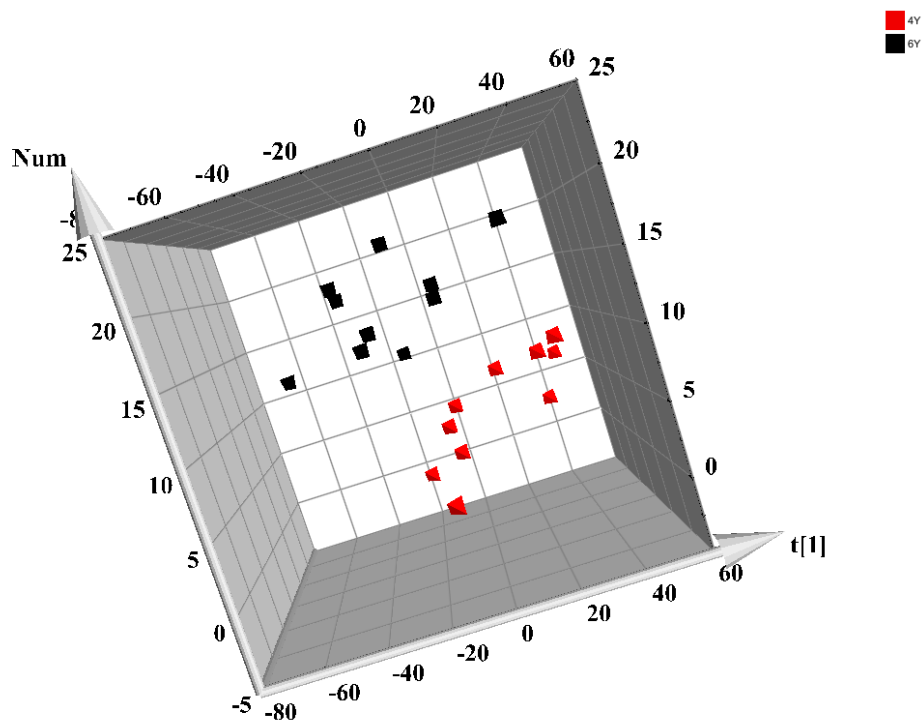


Fig.27 PCA/score plots of processed ginseng sample cultivated for 4 years (4Y) and 6 years (6Y)

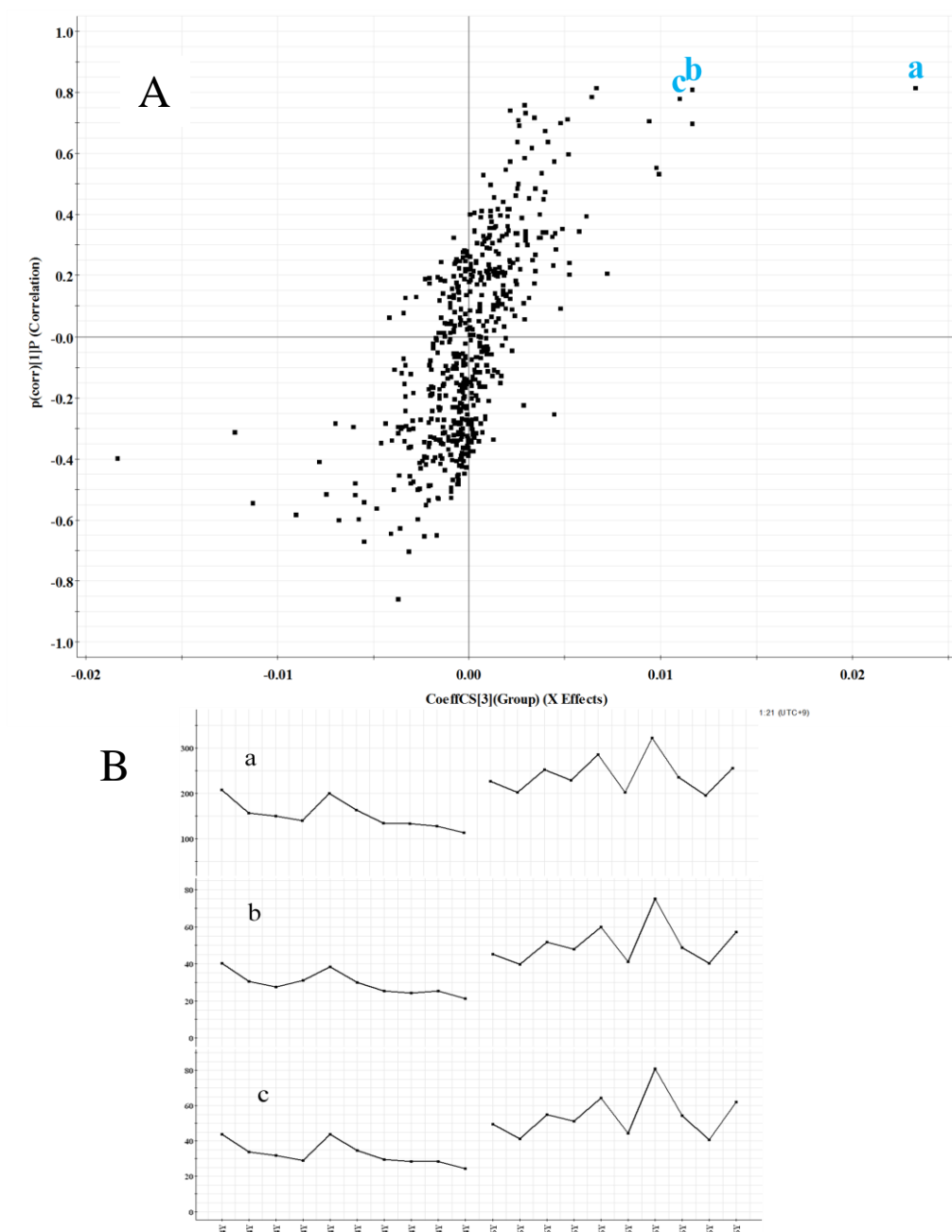


Fig.28 OPLS-DA/S-plots (A) and selected ion intensity trend plots (B) of processed ginseng with different cultivation period. **a** (t_R 14.84 min, m/z 1149.5836), **b** (t_R 15.64 min, m/z 1149.5976), **c** (t_R 14.84 min, m/z 1193.5848)

As shown in Fig. 28A (S-plot), the first three ions, **a** (t_R 14.84 min, m/z 1149.5836), **b** (t_R 15.64 min, m/z 1149.5976), **c** (t_R 14.84 min, m/z 1193.5848) at the top right corner of the “S” were the ions from 6Y that contributed most to the differences between 4Y and 6Y group. However, there was no ion at the lower left corner of the “S”, which is from 4Y group that contributed most to the differences between the two groups.

The ion intensity trends of these ions in the tested samples are provided in Fig. 28 B. The intensities of ions **a**, **b** and **c** were detected all 4Y and 6Y group and relatively higher all 6Y group samples than in the FG group. All three ions could not be confirmed by standard material. But it can be assumed that the ion **a** and **c** was $[M-H-CO_2]^-$ and $[M-H]^-$ of malonyl ginsenoside Rb1, based on exact mass and previous studies. (Sun BS. *et al.*, 2012, Geoffrey CK. *et al.*, 2003, Christensen LP. 2008, Xiu SM., 2002)

3.5 Targeted analysis

To confirm nontargeted analysis, malonyl ginsenoside Rb1 was determined by indirect base catalyzed-hydrolysis method as reported because there was no available commercial reference standard. (William AC. *et al.*, 1996). The UPLC chromatograms of before and after base catalyzed-hydrolysis are shown in Fig. 29. The contents of the five malonyl ginsenosides were represented in Table 13.

The contents of the malonyl ginsenosides in 6Y group were high than in 4Y group. And it showed malonyl ginsenoside Rb1 is most abundant malonyl ginsenoside in five malonyl ginsenosides. Also, this supported nontarget analysis results.

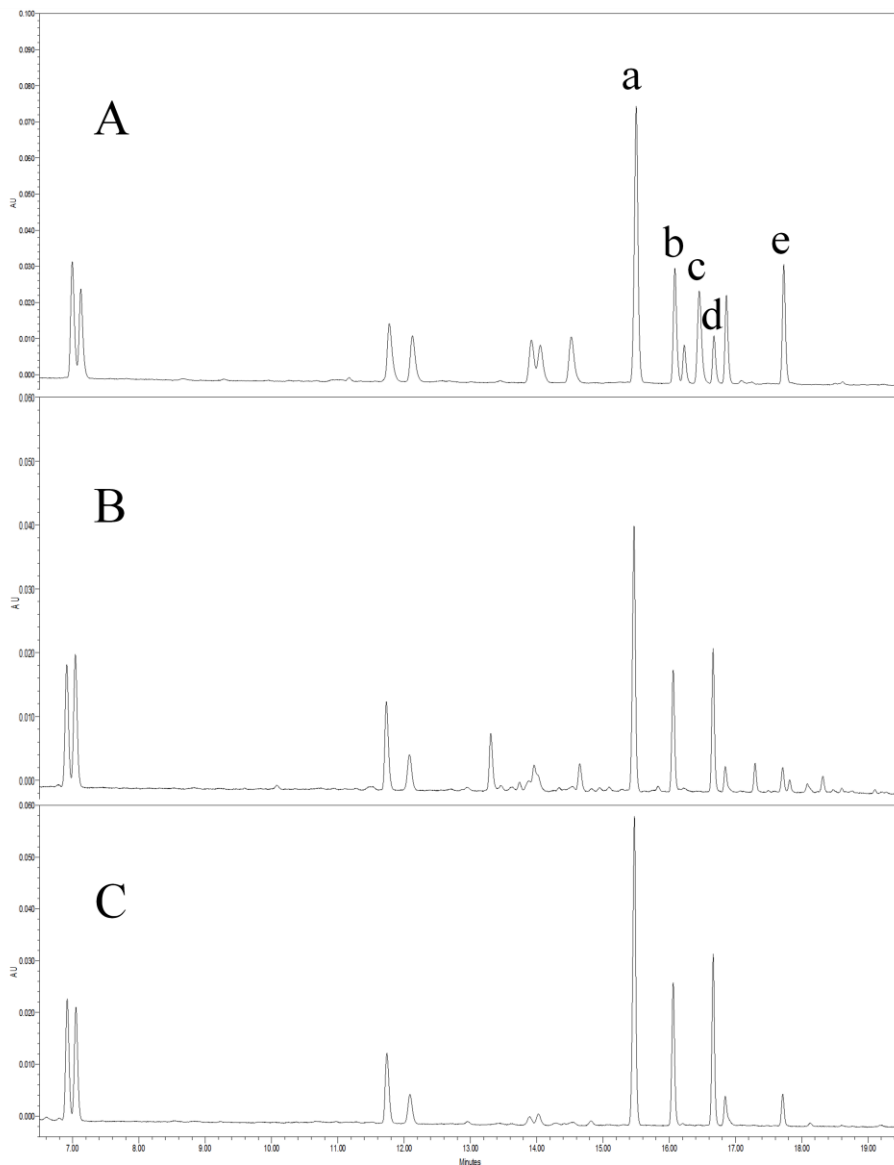


Fig.29 UPLC chromatograms of ginsenoside standard (A), and processed ginseng sample before hydrolysis (B) and after hydrolysis (C). a : ginsenoside Rb1, b : ginsenoside Rc, c : ginsenoside Rb2, d : ginsenoside Rc, e : ginsenoside Rd

Table.13 The contents of malonyl ginsenosides in processed ginseng. (n=10)

	Ginsenoside	Ginsenoside	Ginsenoside	Ginsenoside	Ginsenoside
Analytes	malonyl Rb1	malonyl Rc	malonyl Rb2	malonyl Rb3	malonyl Rd
	(mg/g)	(mg/g)	(mg/g)	(mg/g)	(mg/g)
Processed					
ginseng					
Cultivated	1.84 ±0.81	0.60 ±0.24	0.77 ±0.14	0.12 ±0.07	0.24 ±0.11
for 4 year					
Processed					
ginseng					
Cultivated	2.58 ±1.23	0.83 ±0.45	1.14 ±0.75	0.16 ±0.10	0.28 ±0.19
for 6 year					

CONCLUSIONS

From this study, a rapid and simple simultaneous determination method of 30 ginsenosides (ginsenoside Ro, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, 20(*S*)-Rg2, 20(*R*)-Rg2, 20(*S*)-Rg3, 20(*R*)-Rg3, 20(*S*)-Rh1, 20(*S*)-Rh2, 20(*R*)-Rh2, F1, F2, F4, Ra1, Rg6, Rh4, Rk3, Rg5, Rk1, Rb3, Rk2, Rh3, Compound-Y, Compound-K and Notoginsenoside R1) for metabolomic analysis data confirmation and getting basic information of ginseng samples was established. Separation of the 30 ginsenosides was efficiently undertaken on Acquity BEH C-18 column with gradient elution with phosphoric acids. Especially the chromatogram of ginsenoside Ro was dramatically enhanced by adding phosphoric acid. Under optimized conditions, the detection limits were 0.4 ~ 1.7 mg/L and calibration curves of peak area for the 30 ginsenosides were linear over three orders of magnitude with a correlation coefficients greater than 0.999. The accuracy of the method was tested by recovery measurement of the spiked samples which yielded good results of 89-118%. From these results, proposed method may be helpful in the development and quality of *Panax ginseng* preparations because of its wide range of applications due to the simultaneous analysis of many kinds of ginsenosides.

And then, an ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS) based metabolomic approach was developed in different species, processing step and cultivation period, respectively. First, the validity of metabolomic approach method was verified by comparison of targeted analysis and nontargeted metabolomic analysis of

different ginseng species. The currently known biomarkers, such as ginsenoside Rf and pseudoginsenoside F11, were used to attempt to determine the species with an ultra performance liquid chromatography-photo diode array detector (UPLC-PDA). However, this method did not fully confirm the differentiating attributes of the two processed ginseng species. Thus, an optimized UPLC-QTOF based metabolic profiling method was adapted for the analysis and evaluation of the two processed ginseng species. As a result, all known biomarkers were identified by the proposed metabolomic method and additional potential biomarkers were extracted from the huge amounts of global analysis data.

After verification of validity of this metabolomic approach method, the method was applied to discrimination of ginseng, different processing step and different cultivation period, samples.

Metabolomic analysis of *in-situ* steaming process samples, fresh ginseng, steamed ginseng and red ginseng, was performed with established method. It clearly indicates that there are substantial differences in chemical compositions according to steaming process. Four potential markers (ginsenoside 20(S)-Rg2, 20(R)-Rh1, Rh4 and arginyl-fructose-glucose) for chemical changes in steaming process were identified. Targeted analysis of arginyl-fructose-glucose and malonyl ginsenosides represent arginyl-fructose-glucose (AFG) generated in steaming process. There was no AFG in fresh ginseng and the average contents of AFG in SG and RG group is 26.15 ± 6.99 and 65.90 ± 13.10 mg/g, respectively. These findings were in good agreement with previous nontargeted analysis result. Additionally, it was confirmed that malonyl ginsenosides contents were higher in fresh ginseng samples than in steamed ginseng and red

ginseng. Ginsenoside malonyl Rb1 is most abundant malonyl ginsenoside in all groups.

Finally, metabolomic analysis of fresh ginseng roots by GC-MS clearly indicates that there are substantial differences in non-polar metabolites compositions according to cultivation period (4-6 years). Concentrations of non-polar metabolites from 6 years old root contains higher amount of metabolites than those of 4 years samples and age dependent changes of root growth may cause the metabolic differentiation. PCA of non-polar metabolites showed clear separation between 4 and 6 years old fresh ginseng. Major constituents in non-polar fraction were polyacetylenes (30%), free fatty acids (38%), and monoacyl glycerols (17%). Short chain organic acids, terpenes, and plant steroid comprised approximately 3% of total metabolites. Correlation analysis between metabolites suggested that some fatty acids (C18:2) and monoacylglycerol are important precursors of ginseng polyacetylenes. In addition, strong positive correlation between dehydrocrepenynic acid and others polyacetylenes precursors indicates that polyacetylene biosynthesis in ginseng root may follow the previously reported pathways in other Araliaceae plants.

In addition, UPLC-QTOF MS based metabolomic approach was performed to differentiate of processed ginseng (red ginseng) with different cultivation period (4 and 6 year). Multivariate analysis, including PCA and OPLS-DA of metabolites showed clear separation between 4 and 6 years old ginseng roots. Ginsenoside malonyl Rb1 was confirmed that important metabolites of this differentiation. From targeted analysis, it was confirmed that ginsenoside malonyl Rb1 contents were high in all of the 6 years cultivation samples than in 4 years samples. The average contents of malonyl ginsenoside were 1.84 and

2.58 mg/g at 4 year and 6 year group respectively.

REFERENCES

- Aihua Z, Hui S, Zhigang W, Wenjun S, Ping W, Xijun W. 2010, Metabolomics : Towards understanding traditional Chinese medicine. *Planta Med.* 76, 2026-2035
- Arrhenius, S. 1907, Immunochemie, Akademische Verlagsgesellschaft, Leipzig
- Aslam M, Dent A. 1998, Bioconjugation; Protein coupling techniques for the biomedical sciences, McMillan Reference LTD.: London, UK. pp 50-100
- Asthaagiri A. R, Nelson C. M, Horwitz A. F, Lauffenburger D. A. 1999, Quantitative Relationship among Integrin-Ligand Binding, Adhesion, and Signaling via Focal Adhesion Kinase and Extracellular Signal-regulated Kinase 2, *J. Biol. Chem.* 274, 27119-27127
- Avermeas S, Ternynck T, Guesdon J. L. 1978, Coupling of enzyme to antibodies and antigens, *Scandinavian J. Immunol. Suppl.*, 8(7), 7-23
- Axen, U. 1974, N,N'-carbonyldiimidazole as coupling reagent for the preparation of bovine serum albumin conjugates. *Prostaglandin.* 5, 45-47
- Baeg IH, So SH. 2013, The world ginseng market and the ginseng (Korea). *J Ginseng Res.* 37, 1-7.

Baek SH, Bae ON, Park JH. 2012, Recent methodology in ginseng analysis. *J Ginseng Res.* 36 (2), 119-134

Banerjee B. D. 1987, Development of an Enzyme-linked Immunosorbent Assay for the Quantification of DDA (2,2-bis (*p*-Chlorophenyl) Acetic Acid) in Urine. *Bull. Environ. Contam. Toxicol.* 38, 798

Baranska M, Schultz H, Baranski R, Nothnagel T, Christensen L. P. 2005, In situ simultaneous analysis of polyacetylenes, carotenoids, and polysaccharides in carrot roots. *J. Agric. Food Chem.* 53, 6565-6571

Beasley H. L, Phongkham T, Dqunt M. H, Guihot S. L, Skerritt, J. H. 1998, Development of a panel of immunoassays for monitoring DDT, its metabolites, and analogues in food and environmental matrices. *J. Agric. Food Chem.* 46, 3339-3352

Beasley H. L, Pasha A, Guihot S. L, Skerritt J. H. 2000, An enzyme immunoassay for the organochlorine insecticide hexachlorocyclohexane (HCH), through conversion to trichloropehnols. *Food Agric. Immunol.* 12, 203-215

Bekheit H. K. M, Lucas A. D, Szurdoki F, Gee SJ, Hammock B. D. 1993, An Enzyme Immunoassay for the Environmental Monitoring of the Herbicide Bromacil. *J. Agric. Food Chem.* 41, 2220–2227

Bictash M, Ebbels TM, Chan Q, Loo RL, Yap Ivan KS, Brown IJ, Iorio MD, Daviglus ML, Holmes E, Stamler J, Nicholson JK, Elliott P. 2010, Opening up the “Black Box”: Metabolic phenotyping and metabolome-wide association studies in epidemiology. *J Clin Epidemiol.* 263, 970-979.

Bombardelli E, Bonati A, Gabetta B, Martinelli EM. 1980, Gas-liquid chromatographic method for determination of ginsenosidea in *Panax ginseng*. *J Chromatogr.* 196, 121-132

Bonwick G. A, Putman M, Baugh P. J, Smith C. J, Armitage R, Davies, D. 1994, Immunoassay Development for Permethrin Residues. *Food Agric. Immunol.* 6, 341

Cevallos JM, Corcuera JIR-D, Etxeberria E, Danyluk MD, Rodrick GE. 2009, Metabolomics in food science: a review. *Trends Food Sci Tech.* 20, 557-566

Chan TWD, But PPH, Cheng SW, Kwok IMY, Lau FW, Xu HX. 2000, Differentiation and authentication of *Panax ginseng*, *Panax quinquefolius*, and ginseng products by using HPLC/MS. *Anal Chem.* 72, 1281-1287

Chang YS, Seo EK, Gyllenhaal C, Block KI. 2003, *Panax ginseng*: A role in cancer therapy? *Integr Cancer Ther.* 2, 13-33

Cho EJ, Piao XL, Jang MH, Baek SH, Kim HY, Kang KS, Kwon SW, Park JH. 2008, The effect of steaming on the free amino acid contents and antioxidant activity of *Panax ginseng*. *Food Chem.* 107, 876-882.

Choi KT. 2008, Botanical characteristics, pharmacological effects and medicinal components of Korean *Panax ginseng* CA Meyer. *Acta Pharmacol Sin.* 29, 1109-1118

Choi SJ, Kim TH, Shin YK, Lee CS, Park MJ, Lee HS, Song JH. 2008, Effects of a polyacetylene from *Panax ginseng* on Na⁺ currents in rat dorsal root ganglion neurons. *BRAIN RESEARCH.* 1191. 75-83

Christensen LP. 2008, Ginsenoside: Chemistry, Biosynthesis, Analysis and Potential Health Effects. *Adv Food Nutr Res.* 55, 1–99

Christensen, L. P, Brandt, K. 2006, Bioactive polyacetylenes in food plants of the Apiaceae family: occurrence, bioactivity, and analysis. *J. Pharm. Biomed. Anal.* 41, 683-693

Chu C, Xu S, Li X, Yan J, Liu L. 2012, Profiling the ginsenosides of three ginseng products by LC-Q-Tof/MS. *J Food Sci.* 78, 653-659

Chung IM, Kim JW, Seguin P, Jun YM, Kim SH. 2012, Ginsenosides and phenolics in fresh and processed Korean ginseng (*Panax ginseng* C.A. Meyer): Effects of cultivation location, year, and storage period. *Food Chem* 130, 73-83

Chung WC, Sheu SJ. 1994, Determination of ginsenosides in crude extracts by high-performance liquid chromatography. *J Chromatogr A*. 685, 243-251

Court WA, Hendel JG, Elmi J. 1996, Reversed-phase high-performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*. *J Chromatogr A*. 755, 11-17

Cui JF. 1995, Identification and quantification of ginsenosides in various commercial ginseng preparations. *Eur J Pharm Sci*. 3, 77-85

Dan M, Su M, Gao X, Zhao T, Zhao A, Xie G, Qiu Y, Zhou M, Liu Z, Jia W. 2008, Metabolite profiling of *Panax notoginseng* using UPLC-ESI-MS. *Phytochemistry*. 69, 2237-2244

Du XW, Wills RBH, Stuart DL. 2004, Changes in neutral and malonyl ginsenosides in American ginseng (*Panax quinquefolium*) during drying, storage and ethanolic extraction. *Food Chem* 86, 155-159

Eisenreich, W.; Bacher, A. 2007, Advances of high-resolution NMR techniques in the structural and metabolomic analysis of plant biochemistry. *Phytochemistry*. 68, 2799-2815

Ermer J. Validation in pharmaceutical analysis. 2001, Part I: An integrated approach. *J Pharm Biomed Anal*. 24, 755–767

Fuzzati N. 2004, Analysis methods of ginsenosides. *J Chromatogr B*. 812, 119-133

Geoffrey CK, Melanie-Jayne R, Howes, Christine JL, Monique S. J. Simmonds. 2002, Liquid chromatography/mass spectrometry of malonyl-ginsenosides in the authentication of ginseng. *Rapid Commun. Mass spectrum*. 17, 238-244

Guan J, Lai CM, Li SP. 2007, A rapid method for the simultaneous determination of 11 saponins in *Panax notoginseng* using ultra performance liquid chromatography. *J Pharm Biomed Anal*. 44, 996–1000

Ha YW, Lim SS, Ha IJ, Na YC, Seo JJ, Shin H, Son SH, Kim YS. 2007, Preparative isolation of four ginsenosides from Korean red ginseng (steam-treated *Panax ginseng* C. A. Meyer) by high-speed counter-current chromatography coupled with evaporative light scattering detection. *J Chromatogr A*. 1151, 37-44

Hansen, L.; Boll, P. M. 1986, Polyacetylenes in Araliaceae: their chemistry, biosynthesis, and biological significance. *Phytochemistry*. 25, 285-293

In G, Ahn NG, Bae BS, Han ST, Noh KB, Kim CS. 2012, New method for simultaneous quantification of 12 ginsenosides in red ginseng powder and extract: In-house method validation. *J Ginseng Res*. 36, 205-210

Jan L, Nicolas S, Joachim K, Lothar W, Alisdair RF. 2006, Gas chromatography mass spectrometry-based metabolite profiling in plants. *NATURE PROTOCOLS*. 1(1), 387-396

Jung DW, Lee JM, Sung CK. 2002, Enzyme-linked immunosorbent assay for the determination of 20(*S*)-protopanaxatriol. *Anal Chim Acta*. 462, 157-163

Jung MY, Jeon BS, Bock JY. 2002, Free, esterified, and insoluble-bound phenolic acids in white and red Korean ginsengs (*Panax ginseng* C.A. Meyer). *Food Chem*, 79, 105-111

Kanazawa H, Nagata Y, Matsushima Y, Tomoda M. 1990, Simultaneous determination of ginsenosides and saikosaponins by high-performance liquid chromatography. *J Chromatogr*. 507, 327-332

Kanazawa H, Nagata Y, Matsushima Y, Tomoda M. 1993, Determination of acidic saponins in crude drugs by high-performance liquid chromatography on octadecylsilyl porous glass. *J Chromatogr*. 630, 408-414

Kang JH, Lee SY, Kang SM, Kwon HN, Park JH, Kwon SW, Park SH. 2008, NMR-based metabolomics approach for the differentiation of ginseng(*Panax ginseng*) roots from different origins. *Arch Pharm Res*. 31, 330-336

Kang KS, Yamabe N, Kim HY, Okamoto T, Sei Y, Yokozawa T. 2007, Increase in the free radical scavenging activities of American ginseng by heat processing and its safety evaluation. *J ethnopharmacol.* 113, 225-232

Kim HK, Choi YH. 2011, Verpoorte R. NMR-based plant metabolomics: where do we stand, where do we go? *Trends Biotechnol.* 29, 267–275

Kim NH, Kim KO, Choi BY, Lee DH, Shin YS, Bang KH, Cha S-W, Lee JW, Choi H-K, Jang DS, Lee DH. 2011, Metabolomic approach for age discrimination of panax ginseng using UPLC-Q-Rof MS. *J Agric Food Chem.* 59, 10435-10441

Kim NH, Kim KO, Lee DH, Shin YS, Bang KH, Cha SW, Lee JW, Choi HK, Hwang BY, Lee DH. 2012, Nontargeted metabolomics approach for age differentiation and structure interpretation of age-dependent key constituents in hairy roots of panax ginseng. *J. Nat. Prod.* 1777-1784

Kim DH. 2012. Chemical diversity of *Panax ginseng*, *Panax quinquefolium*, and *Panax notoginseng*, *J. Ginseng Res.* 36. 1-15

Kim NH, Kim KO, Lee DH, Shin YS, Bang KH, Cha S-W, Lee JW, Choi H-K, Hwang BY, Lee DH. 2012, Nontargeted metabolomics approach for age differentiation and structure interpretation of age-dependent key constituents in hairy roots of *panax ginseng*. *J Nat Prod.* 75, 1777-1784

Kim SN, Ha YW, Shin H, Son SH, Wu SJ, Kim YS. 2007, Simultaneous quantification of 14 ginsenosides in *Panax ginseng* C.A. Meyer (Korean red ginseng) by HPLC-ELSD and its application to quality control. *J Pharm Biomed Anal.* 45, 164–170

King JY, Ferrara R, Tabibiazar R, Spin JM, Chen MM, Kuchinsky A, Vailaya A, Kincaid R, Tsalenko A, Deng DX, Connolly A, Zhang P, Yang E, Watt C, Yakhini Z, Ben-Dor A, Adler A, Bruhn L, Tsao P, Quertermous T, Ashley E. 2005, Pathway analysis of coronary atherosclerosis. *Physiol. Genomics.* 23, 103-118

Kwon SW, Han SB, Park IH, Kim JM, Park MK, Park JH. 2001, Liquid chromatographic determination of less polar ginsenosides in processed ginseng. *J Chromatogr A.* 921, 335-339

Lau AJ, Seo BH, Woo SO, Koh HL. 2004, High-performance liquid chromatographic method with quantitative comparisons of whole chromatogram of raw and steamed *Panax notoginseng*. *J Chromatogr A.* 1057, 141-149

Lau AJ, Woo SO, Koh HL. 2003, Analysis of saponins in raw and steamed *Panax notoginseng* using high-performance liquid chromatography with diode array detection. *J Chromatogr A.* 1011, 77-87

Lee SA, Jo HK, Im BO, Kim SG, Whang WK, Ko SK. 2012, Changes in the contents of prosapogenin in the red ginseng (*Panax ginseng*) depending on steaming batches. *J Ginseng Res.* 36 (1), 102-106

Lee SI, Kwon HJ, Lee YM, Lee JH, Hong SP. 2012, Simultaneous analysis method for polar and non-polar ginsenosides in red ginseng by reversed-phase HPLC-PAD. *J Pharm Biomed Anal.* 60, 80–85

Lee YJ, Kim HY, Kang KS, Lee JG, Yokozawa T, Park JH. 2008, The chemical and hydroxyl radical scavenging activity changes of ginsenoside-Rb₁ by heat processing. *Bioorg Med Chem Lett.* 18, 4515-4520

Lee, SM. Bae KH. Sohn HJ. 2009, Panaxfuraynes A and B, two new tetrahydrofuranic polyacetylene glycosides from *Panax ginseng* C. A. Meyer. *Tetrahedron Lett.* 50, 416-418

Leung Kelvin SY, Chan K, Bensoussan A, 2007, Munroe MJ. Application of atmospheric pressure chemical ionization mass spectrometry in the identification and differentiation of *Panax* species. *Phytochem Anal.* 18, 146-150.

Li L, Luo GA, Liang QL, Hu P, Wang YM. 2010, Rapid qualitative and quantitative analyses of Asian ginseng in adulterated American ginseng preparations by UPLC/Q-TOF-MS. *J Pharm Biomed Anal.* 52, 66–72

Lin WN, Lu HY, Lee MS, Yang SY, Chen HJ, Chang YS, Chang WT. 2010, Evaluation of the cultivation age of dried ginseng radix and its commercial products by using ^1H -NMR fingerprint analysis. *Am J Chinese Med.* 38, 205-218

Lizhi Z, Robert EM. 2001, Improved syntheses of methyl (14E)- and (14Z)-dehydrocrepenynate: key intermediates in plant and fungal polyacetylene biosynthesis. *Tetrahedron Letters.* 42, 3803-3805

Liu JH, Lee CS, Leung KM, Yan ZK, Shen BH, Zhao ZZ, Jiang ZH. 2007, Quantification of two polyacetylenes in radix ginseng and roots of related *panax* species using a gas chromatography-mass spectrometric method. *J. Agric. Food Chem.* 55, 8830-8835

Madsen R, Lundstedt T, Trygg J. 2010, Chemometrics in metabolomics-A review in human disease diagnosis. *Anal Chim Acta.* 659, 223-33

Mattoli L, Cangi F, Maidecchi A, Ghiara C, Ragazzi E, tubaro M, Stella L, Tisato F, Traldi P. 2006, Metabolomic fingerprinting of plant extracts. *J. Mass Spectrom.* 41, 1534-1545

Minto RE, Blacklock B. 2008 J. Biosynthesis and function of polyacetylenes and allied natural products. *Prog. Lipid. Res* 47, 233-306

Nadezhda A, Hong-Wei K. Rob V. D. H, Shih-Ying Y, Young H. C, Hye K. K, Mei W, Thomas H, Jan V. D. G, Guowang X. Rob V. 2008, Recent methodology in the phytochemical analysis of ginseng. *Phytochem. Anal.* 19, 2-16

Nho KB, Sohn HJ. 1989, Comparative studies on methods of extracting polyacetylene compounds from white ginseng. *Korean J. Ginseng Sci* 13, 198-201.

Nishi H, Terabe S. 1996, Micellar electrokinetic chromatography perspectives in drug analysis. *J Chromatogr A.* 735, 3-27.

Nomar EW, Park HM, Ryu KB, Kim JH, Yang TJ, Kim HH. 2012, Karyotype analysis of *Panax ginseng* C. A. Meyer, 1843 (Araliaceae) based on rDNA loci and DAPI band distribution. *CompCytogen.* 6(4), 425-441

Novakova L, Matysova L, Solich P. 2006, Advantages of application of UPLC in pharmaceutical analysis. *Talanta.* 68, 908-918

Oleszek WA, Bialy Z. 2006, Chromatographic determination of plant saponins- An update (2002-2005). *J Chromatogr A.* 1112, 78-91

Oleszek WA. 2002, Chromatographic determination of plant saponins. *J Chromatogr A.* 967, 147-162.

Park HW, In G, Lee MW, Kim SY, Kim KT, Cho BG, Han GH, Chang IM. 2013, Simultaneous determination of 30 ginsenosides in *Panax ginseng* preparations using ultra performance liquid chromatography. *J Ginseng Res.* 37, 457-467

Park JD. 1996, Recent studies on the chemical constituents of Korean ginseng. *Korean J Ginseng Sci.* 20, 389-415

Park JI, Han SB, Kim JM, Piao L, Kwon SW, Lim NY, Kang TL, Park MK, Park JH. 2002, Four new acetylated ginsenosides from processed ginseng (Sun Ginseng). *Arch Pharm Res.* 25, 837-841

Park MK, Park JH, Han SB, Shin YG, Park IH. 1996, High-performance liquid chromatographic analysis of ginseng saponins using evaporative light scattering detection. *J Chromatogr A.* 736, 77-81.

Putri SP, Nakayama Y, Matsuda F, Uchikata T, Kobayashi S, Matsubara A, Fukusaki E. 2013, Current metabolomics: Practical applications. *J Biosci Bioeng.* 115, 579-589

Qiu Y, Lu X, Pang T, Ma C, Li X, Xu G. 2008, Determination of radix ginseng volatile oils at different ages by comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry. *J. Sep. Sci.* 31, 3451-3457

Richter R, Basar S, Koch A, Konig W. A. 2005, Three sesquiterpene hydrocarbons from the roots of *Panax ginseng* C.A. Meyer (Araliaceae). *Phytochemistry*. 66, 2708-2713

Sanada S, Kondo N, Shoji J, Tanaka O, Shibata S. 1974, Studies on the saponins of ginseng. I. Structures of ginsenoside-Ro, -Rb1, -Rb2, -Rc and -Rd. *Chem Pharm Bull*. 22, 421-428

Shangguan D, Han H, Zhao R, Zhao Y, Xiong S, Liu G. 2001, New method for high-performance liquid chromatographic separation and fluorescence detection of ginsenosides. *J Chromatogr A*. 910: 367-372.

Shi Y, Sun C, Zheng B, Li Y, Wang Y. 2010, Simultaneous determination of nine ginsenosides in functional foods by high-performance liquid chromatography with diode array detector detection. *Food chem*. 123, 1322-1327

Shibata S, Ando T, Tanaka O, Meguro Y, Soma K, Iida Y. 1965, Studies on the constituents of Japanese and Chinese crude drugs. XII. Panaxadiol, a sapogenin of ginseng roots. *Chem Pharm Bull*. 11, 762-765

Shin YS, Bang KH, In DS, Kim OT, Hyun DY, Ahn IO, Ku BC, Kim SW, Seong NS, Cha SW, Lee DH, Choi HK. 2007, Fingerprinting analysis of fresh ginseng roots of different ages using ¹H-NMR spectroscopy and principal components analysis. *Arch Pharm Res*. 30, 1625-1628.

Shyur LF, Yang NS. 2008, Metabolomics for phytomedicine research and drug development. *Curr Opin Chem Biol.* 12, 66-71

Soldati F. 2000, *Panax ginseng*; Standardization and Biological Activity. In: Cutler SJ, Cutler HG. ed. Biologically active natural products. New York: CRC press. 209-232.

Sun BS, Gu LJ, Fang ZM, Wang CY, Lee MR, Li Z, Li JJ, Sung CK. 2009, Simultaneous quantification of 19 ginsenosides in black ginseng developed from *Panax ginseng* by HPLC-ELSD. *J Pharm Biomed Anal.* 50, 15–22

Sun BS. Pan FY. Sung CK. 2011, Repetitious steaming-induced chemical transformations and global quality black ginseng derived from *Panax ginseng* by HPLC-ESI-MS/MSⁿ based chemical profiling approach. *Biotechnology and Bioprocess Engineering.* 16, 956-965

Sun BS, Xu MY, Li Z, Wang YB, Sung CK. 2012, UPLC-Q-TOF-MS/MS analysis for steaming times-dependent profiling of steamed *Panax quinquefolius* and its ginsenosides transformations induced by repetitious steaming. *J Ginseng Res.* 36, 277-290

Sun S, Wang CZ, Tong R, Li XL, Fishbein A, Wang Q, He TC, Du W, Yuan CS. 2010, Effects of steaming the root of *Panax notoginseng* on chemical composition and anticancer activities. *Food Chem.* 118, 307-314

Taverniers I, Loose MD, Bockstaele EV. 2004, Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance.

Trends Anal Chem. 23, 535–552

Toh DF, New LS, Koh HL, Chan Eric CY. 2010, Ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS) for time-dependent profiling of raw and steamed *Panax notoginseng*. *J Pharm Biomed Anal.* 52, 43-50

Wang D, Liao PY, Zhu HT, Chen KK, Xu M, Zhang YJ, Yang CR. 2012, The processing of *Panax notoginseng* and the transformation of its saponin components. *Food Chem.* 132, 1808-1813

Wang X, Zhao T, Gao X, Dan M, Zhou M, Jia W. 2007, Simultaneous determination of 17 ginsenosides in rat urine by ultra performance liquid chromatography-mass spectrometry with solid-phase extraction. *Anal Chim Acta.* 594, 265-273

Wang Y, You J, Yu Y, Qu C, Zhang H, Ding L, Zhang H, Li X. 2008, Analysis of ginsenosides in *Panax ginseng* in high pressure microwave-assisted extraction. *Food Chem.* 110, 161-167

Wei W, Song F, Dianjing G, Jun M, Qiujie Q, Qing Y, Shuying L. 2012, Mass spectrometry-based approach in ginseng research : A promising way to metabolomics, *Current Analytical Chemistry*, 8(1), 43-66

William AC, John GH, Jama E. 1996, Reversed-phase high-performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*.

Journal of Chromatography A. 755, 11-17

William AM, Christian MW. 2013, Simplified ultrasonically- and microwave-assisted solvent extraction for the determination of ginsenosides in powdered *Panax ginseng* rhizomes using liquid chromatography with UV absorbance or electrospray mass spectrometric detection. *Anal Bioanal Chem*, 405, 4511-4522

Wishart DS. 2008, Metabolomics; application to food science and nutrition research. *Trends Food Sci Tech*. 19, 482-493

Xiao JF, Zhou B, Ressom HW. 2012, Metabolite identification and quantitation in LC-MS/MS-based metabolomics. *Trends Anal Chem*. 32, 1–14

Xie G, Plumb R, Su M, Xu Z, Zhao A, Qiu M, Liu Z, Jia W. 2008, Ultra-performance LC/TOF MS analysis of medicinal *Panax* herbs for metabolomic research. *J Sep Sci*. 31, 1015-1026

Xie YY, Luo D, Cheng YJ, Ma JF, Wang YM, Liang QL, Luo GA. 2012, Steaming-induced chemical transformations and holistic quality assessment of red ginseng derived from *panax ginseng* by means of HPLC-ESI-MS/MS based multicomponent quantification fingerprint. *J Agric Food Chem*. 60, 8213-8224

Xiu SM, Chris DM, Chunyan H, Rayomnd EM. 2002, Electrospray ionization mass spectrometry of ginsenosides. *J. Mass Spectrom*, 37, 495-506

Yamaguchi H, Kasai R, Matsuura H, Tanaka O, Fuwa T. 1998, High-performance liquid chromatographic analysis of acidic saponins of ginseng and related plants. *Chem Pharm Bull*, 36, 3468-3473

Yang SO, Lee SW, Kim YO, Sohn SH, Kim YC, Hyun DY, Hong YP, Shin YS. 2013, HPLC-based metabolic profiling and quality control of leaves of different *Panax* species. *Journal of Ginseng Research*, 37(2), 248-253

Yang SO, Shin YS, Hyun SH, Cho SY, Bang KH, Lee DH, Choi SP, Choi HK. 2012. NMR-based metabolic profiling and differentiation of ginseng roots according to cultivation ages. *Journal of Pharmaceutical and Biomedical Analysis*, 58. 19-26

Yizhen Z, Xunan L, Tao L, Jianping L, Wenju Z, Qing M. 2013, Analysis of nonpolar components from ginseng of different age. *American Journal of Plant Sciences*, 4, 92-97.

Yun TK. 2001, Brief introduction of *Panax ginseng* C. A. Meyer. *J Korean Med Sci*. 16(Suppl), S3-5

국 문 요 약

인삼 연구에 UPLC-QTOF MS (ultraperformance liquid chromatography-quadrupole time of flight mass spectrometry)를 활용한 대사체학 연구기법을 적용하기 위하여 서로 다른 종 (*Panax ginseng*, *Panax quinquefolius*)의 홍삼을 대상으로 그 방법의 타당성을 검증하였으며 이를 위하여 UPLC (ultraperformance Liquid chromatography)를 이용한 진세노사이드의 동시분석법 또한 확립하였다. 30 종의 진세노사이드를 분리하기 위하여 이동상 조건을 최적화하고 분석법의 타당성을 검증하였고 분석법의 검출한계 및 회수율은 각각 0.4 ~ 1.7 mg/L, 89-118 % 이었다.

서로 다른 종의 홍삼의 대사체 분석결과 잘 알려진 마커 물질인 ginsenoside Rf 및 pseudoginsenoside F11 을 도출하였으며, 추가적으로 20-gluco-ginsenoside Rf 역시 확인되어 본 대사체 연구 기법이 타당한 결과를 도출함을 확인하였다.

이렇게 검증된 방법을 활용하여 인삼의 가공과정 (수삼, 찢삼, 홍삼) 중 성분차이를 *In-situ* 상태의 시료를 채취하여 확인하였다. 주성분 분석결과 세 그룹의 시료가 명확하게 나뉘었으며 이를 통하여 세 그룹간 성분에 큰 차이가 있음을 확인하였다. 차이를 나타내게 하는 마커 물질로 ginsenoside 20(S)-Rg2, 20(R)-Rh1, Rh4 and arginyl-fructose-glucose (AFG) 네 성분을 도출하였다. 또한 AFG 의 targeted 분석을 통하여 가공과정 중 AFG 의 함량이 증가하는 것을 검증하였다. 수삼에는 AFG 가 존재하지 않았으며 찢삼 및 홍삼에서의 함량은 각각 26.15 ± 6.99 및 65.90 ± 13.10 mg/g 이었다.

수삼의 재배기간 (4년 및 6년)에 따른 성분 차이는 GC-MS를 이용한 대사체 프로파일링을 통하여 확인하였으며, 비극성 대사체의 주성분 분석결과 4년근과 6년근이 나뉘어짐을 확인할 수 있었다. 주요 성분으로는 polyacetylenes (30%), free fatty acids (38%), monoacyl glycerols (17%) 그리고 short chain organic acids, terpens, plant steroid 등이 약 3% 정도를 차지하는 것으로 확인되었다. 대사체간 상관관계 분석을 통하여 linoleic acid와 monoacylglycerol이 polyacetylene의 주요한 전구체임을 확인하였다. 추가적으로 dehydrocrepenynic acid와 다른 polyacetylene 전구체들과의 양의 상관관계를 보였으며 이는 인삼에서의 polyacetylene 생합성 과정이 이전에 보고된 Araliaceae 과 식물에서의 경로를 따르는 것으로 판단된다.

마지막으로 재배기간 (4년 및 6년)이 다른 수삼으로 가공한 홍삼의 성분 차이를 UPLC-QTOF MS를 이용한 대사체학 기법으로 확인하였으며, 주성분 분석결과 역시 4년근 및 6년근 홍삼, 두 그룹으로 나뉘는 것을 확인하였다. 마커물질로는 ginsenoside malonyl Rb1이 도출되었으며 targeted 분석결과 ginsenoside malonyl Rb1의 함량은 4년근과 6년근에서 각각 1.84 및 2.58 mg/g으로 확인되었다.

주요어 : 대사체학, UPLC-QTOF MS, GC-MS, 인삼 종, 홍삼가공과정, 연근

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PUBLICATIONS

1. 문준관, **박희원**, 최훈, 홍용순, 류광현, 이윤형, 이규승, 김정한. 2003. 포도 중 살충제 Fenitrothion 의 잔류 특성, *HORTICULTURE ENVIRONMENT AND BIOTECHNOLOGY*, 44(4), 497-502
2. YS Hong, **HW Park**, H Choi, JK Moon MJ Kim, JUK Kim, YD Lee, CH Oh, JH Kim. 2004. An Improved Method for Multiresidue Analysis of Pesticides in Lettuce, Chinese cabbage and Green pepper by Gas Chromatography, *Korean journal of Environmental Agriculture*, 23(3), 158-169
3. JY Byoun, H Choi, JK Moon, **HW Park**, KH Liu, YB Ihm, BSoo Park, JH Kim. 2005. Risk Assessment of Human Exposure to Methidathion during Harvest of Cucumber in Green House. *Journal of Toxicology and Public Health*, 21(4), 297-301
4. H Choi, JK Moon, KH Liu, **HW Park**, YB Ihm, BS Park, JH Kim. 2006. Risk Assessment of Human Exposure to Cypermethrin During Treatment of Mandarin Fields, *Archives of Environmental Contamination and Toxicology*, 50, 437-442,
5. HK Lee, JK Moon, CH Chang, H Choi, **HW Park**, BS Park, HS Lee, EC Hwang, YD Lee, KH Liu, JHan Kim, 2006. Stereoselective metabolism of Endosulfan by Human Liver Microsomes and Human Cytochrome P450 Isiforms, *Drug Metabolism and Disposition*, 34, 1090-1095

6. 이종화, **박희원**, 금영수, 권찬혁, 이영득, 김정한. 2008. 시설 내 오이 재배 중 살균제 Boscalid 의 잔류특성, *The Korean Journal of Pesticide Science*. 12(1). 67-73

7. 이혜리, 류명주, **박희원**, 나예림, 송혁환, Zhu youngzhe, 금영수, 김정한. 2009. HPLC 를 이용한 배추, 사과, 감귤, 고추 중 살균제 Fenhexamid 의 정밀분석법, *The Korean Journal of Pesticide Science*, 13(4), 223-231

8. 김성우, 이은미, Yang Lin, **박희원**, 이혜리, 류명주, 나예림, 노재억, 금영수, 송혁환, 김정한. 2009. 포도의 재배기간 중 살충제 bifenthrin 의 생산단계 농약잔류허용기준의 설정, *The Korean Journal of Pesticide Science*, 13(4), 241-248

9. 이은미, 이혜리, 류명주, **박희원**, 나예림, 송혁환, 금영수, Zhu youngzhe, 김정한. 2009. GC-ECD 를 이용한 배추, 사과, 감귤, 고추, 현미 중 살균제 Prochloraz 의 분석법, *Korean Jorunal of Environmental Agriculture*, 28(4), 427-434

10. YS Keum, HR Lee, **HW Park**, JHan Kim. 2010. Biodegradation of bisphenol A and its halogenated analogues by Cunninghamella elegans ATCC36112. *Biodegradation*, 21(6), 989-997

11. YZhe Zhu, YS Keum, L Yang, HR Lee, **HW Park**, JH Kim. 2010. Metabolism of a Fungicide Mepanipyrim by Soil Fungus Cunninghamella elegans ATCC36112. *Journal of Agricultural and Food Chemistry*. 58(23), 12379-12384

12. 이영주, **박희원**, 문준관, 최홍림, 김정한. 2010. 돈분액비 및 가공돈분액 처리에 따른 발토양과 논토양 중 살충제 Diazinon 의 분해 특성, *The Korean Journal of Pesticide Science*, 14(4), 332-338
13. H Choi, JK Moon, BS Park, **HW Park**, SY Park, TS Kim, DH Kim, TH Ryu, SJ Kweon, JH Kim. 2012. Comparative Nutritional Analysis for Genetically Modified Rice, Iksan483 and Milyang204, and Nontransgenic Counterparts, *J Korean Soc Appl Biol Chem.*, Vol.55(1), 19-22
14. YS Keum, **HW Park**, HH Song, BD Kim, BCI Kang, JH Kim. 2012. Metabolite Analysis of Long Chain Branched Fatty Acids and Capsaicin Biosynthesis in Capsicumannuum placenta, *J Korean Soc Appl Biol Chem.*, Vol.55(2), 189-195
15. **HW Park**, G In, ST Han, MW Lee, KT Kim, BG Cho, GH Han, IM Chang. 2013. Simultaneous determination of 30 ginsenosides in Panax ginseng preparations using ultra performance liquid chromatography, *Journal of Ginseng Research*, 37(4), 457-467
16. **HW Park**, G In, JH Kim, BG Cho, GH Han, IM Chang. 2014. Metabolomic approach for discrimination of processed ginseng genus (Panax ginseng and Panax quinquefolius) using UPLC-QTOF MS, *Journal of Ginseng Research*, 38(1), 59-65

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