Journal of Pharmaceutical and Biomedical Analysis 64–65 (2012) 56–63

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

The effects of dynamic changes of malonyl ginsenosides on evaluation and quality control of *Panax ginseng* C.A. Meyer

Zhi Liu[a,b,c], Yu Li[b], Xiang Li[d], Chang-Chun Ruan[a,c], Li-Juan Wang[a], Guang-Zhi Sun[a,*]

[a] Institute of Agricultural Modernization, Jilin Agricultural University, Changchun 130118, China
[b] Engineering Research Center of Chinese Ministry of Education for Edible and Medicinal Fungi, Jilin Agricultural University, Changchun 130118, China
[c] Engineering Research Center of Natural Enemy Insects, Jilin Agricultural University, Changchun 130118, China
[d] College of Plant Science, Jilin University, Changchun 130062, China

ARTICLE INFO

Article history:
Received 14 November 2011
Received in revised form 5 February 2012
Accepted 7 February 2012
Available online 15 February 2012

Keywords:
Panax ginseng
Malonyl ginsenosides
Evaluation
Quality control
HPLC-UV

ABSTRACT

To clarify the effects of malonyl ginsenosides (MGR) on evaluation and quality control of *Panax ginseng*, the contents of neutral and malonyl ginsenosides from *P. ginseng* were examined by high-performance liquid chromatography equipped with UV-VIS detector (HPLC-UV) during extraction, processing and storage. Several solvents, including water, ethanol, methanol, and *n*-butanol were used in the cold-soaked extraction (CSE). Among the four extraction solvents, methanol was found to be the most efficient. CSE was compared with other extraction methods such as Soxhlet extraction (SE), heat reflux extraction (HRE), ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE). The content of MGR showed significant differences, higher in CSE and UAE; lower in MAE and HRE; no MGR could be detected after SE. However, the total contents of neutral and malonyl ginsenosides were not different. Meanwhile, white ginseng, stored at 25 °C in air of low humidity, showed a marked decrease in the concentration of MGR from 1.19% to 0.63% but with an increase in the neutral ginsenosides from 1.12% to 1.53% after 0–9-month storage. The results indicated that MGR changed dynamically in *P. ginseng* with different extraction solvents, extraction methods and increasing storage time. The total ginsenosides was not only underestimated but also determined imprecisely by ignoring malonyl ginsenosides. On the basis of our results, we suggest that malonyl ginsenosides should be transformed into the corresponding neutral ginsenosides during sample preparation for quality control and evaluation of *P. ginseng*. Then the content of six neutral ginsenosides in samples was used as the true level of total ginsenosides. The results reported here might provide useful information for accurate evaluation and quality control of *P. ginseng*.

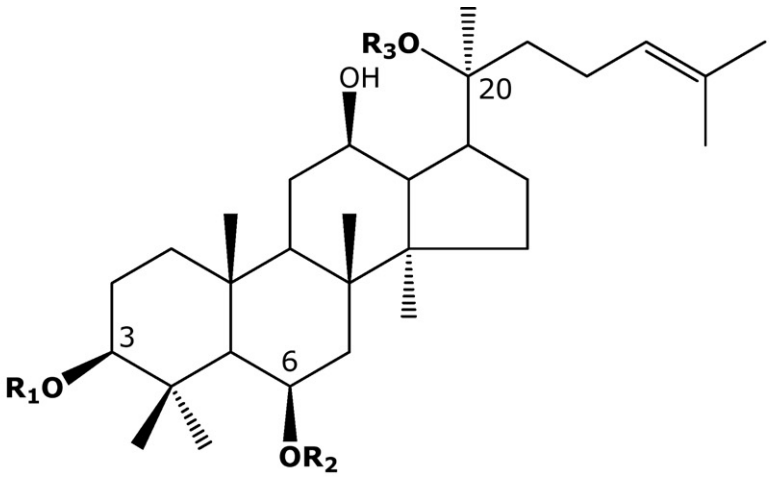
1. Introduction

Ginseng (*Panax ginseng* C.A. Meyer) has been used in China for thousands of years as a traditional medicine and proved to have wide pharmacological properties, such as tonic, sedative, anti-fatigue, or anti-gastric ulcer drug, antidiabetic and antitumor activities [1–4]. In the recent years, *P. ginseng* roots have been not only used as therapeutic agents but also marketed as dietary supplements and raw materials of health food [5,6]. The active components in ginseng are commonly considered to be ginsenosides, a group of steroidal saponins, including neutral ginsenosides, malonyl ginsenosides, and the oleanolic acid-type ginsenoside (Fig. 1). The six major neutral ginsenosides (Rb_1, Rb_2, Rc, Rd, Re and Rg_1) are frequently used as the main index for ginseng product evaluation and quality control [7–12]. Malonyl ginsenosides, which are thermally unstable, can degrade into corresponding neutral ginsenosides [13,14]. To our knowledge HPLC standard protocols do not normally measure malonyl ginsenosides for two main reasons. First, a phosphate buffer is not used in the mobile phases and secondly malonyl ginsenosides are not easily available. In previous study, the identification of the malonyl ginsenosides was carried out by comparing their chromatographic retention times with published data or by HPLC-MS [6,11,15,16]. The malonyl ginsenosides were also determined with an indirect method [8,17], and HPLC analysis was performed twice for each sample. Therefore, the malonyl ginsenosides were not used as index for evaluation and quality control of ginseng product.

P. ginseng is one of the most sold oriental herbal medicines in the world, and therefore it is important that the national authorities adopt common analytical methodologies in order to establish precise quality standards and have a control on the marketed products. However, concerning the quantitative data, a great variability

* Corresponding author. Tel.: +86 431 8451 0949; fax: +86 431 8451 0949.
E-mail addresses: jlndxdhs@126.com (Z. Liu), liyujlau@gmail.com (Y. Li), xiang.ca@gmail.com (X. Li), ccyuan@yahoo.com (C.-C. Ruan), ljwang@yahoo.com (L.-J. Wang), gzsun1967@yahoo.com (G.-Z. Sun).

0731-7085/$ – see front matter.
doi:10.1016/j.jpba.2012.02.005



Ginsenoside	R1	R2	R3
malonyl-Rb1	glc(2-1)glc-ma	H	glc(6-1)glc
malonyl-Rc	glc(2-1)glc-ma	H	glc(6-1)ara(furan)
malonyl-Rb2	glc(2-1)glc-ma	H	glc(6-1)ara(pyran)
malonyl-Rd	glc(2-1)glc-ma	H	glc
Rb1	glc(2-1)glc	H	glc(6-1)glc
Rc	glc(2-1)glc	H	glc(6-1)ara(furan)
Rb2	glc(2-1)glc	H	glc(6-1)ara(pyran)
Rd	glc(2-1)glc	H	glc
Rg1	H	glc	glc
Re	H	glc(2-1)rha	glc

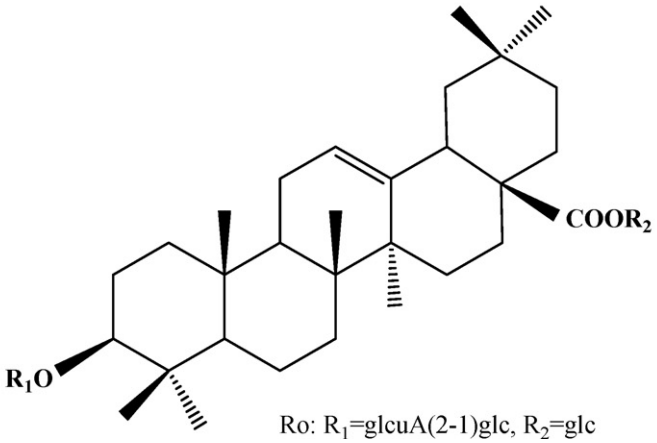


Fig. 1. Structure of the main ginsenosides from the roots of *Panax ginseng*. glc, glucosyl; ma, malonyl; rha, rhamnosyl; ara, arabinosyl; glcuA, glucuronic acid.

in the content of ginsenosides can be found in the literature [18]. For example, total content of ginsenosides in *P. ginseng* varied from 0.2% to 2% in main roots and from 4% to 9% in root hair [19]. This variability in main roots or root hair of ginseng can be partly ascribed to soil, weather conditions, geographical location, nonstandard processing and manufacturing methods. In addition, the variability of individual ginsenoside has been shown to be influenced by genotype and environmental factors. Lim et al. [20] have, for example, shown that the Re content was influenced by genotype, Rb_1, Rc, and Rb_2 contents were influenced by location, and that the content of Rg_1 and Rd were influenced by both genotype and location. The content of malonyl-ginsenoside Rb_1, Rb_2, Rc, and Rd were demonstrated to represent up to 60% of the total content in ginsenosides [21]. Awang commented that the true ginsenosides content of ginseng was underestimated by ignoring malonyl ginsenosides, as they can constitute a substantial proportion of the total ginsenosides

content [22]. However, the published data is still scarce on effects of the content and degradation of malonyl ginsenosides in *P. ginseng* on quality control and evaluation.

Many conventional extraction techniques, such as Soxhlet extraction (SE), reflux extraction (RE), ultrasonic extraction (UE), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) have been used to extract ginsenosides from *P. ginseng* [23–26]. High pressure microwave assisted extraction (HPMAE) and ultrahigh pressure extraction (UPE) are relatively new extraction methods and employed for the extraction of ginsenosides from *P. ginseng* [6,27] which had been shown to enhance the extraction efficiency of the components of interest. However, these extraction methods focused on the extraction efficiency of ginsenosides and ignored malonyl ginsenosides.

Of the commercial ginseng products, red ginseng (RG) is made by steaming and drying fresh ginseng by the traditional method, whereas white ginseng (WG) is made by simply drying the fresh ginseng. In commercial practice, dried ginseng is stored for a long time during marketing or before further processing into food or therapeutic products. It was reported that the unique ginsenosides in RG are ginsenosides Rg_3, Rg_5, Rh_2 and Rk_1 which are less polar than in fresh ginseng [28]. Li et al. reported that malonyl ginsenoside Rb_1 was transformed into ginsenoside Rb_1, Rd, Rg_3 and Rh_2 during the processing of red ginseng [29]. Du et al. reported that neutral and malonyl ginsenosides changed in American ginseng powder after 0–12-week storage [8]. However, these studies usually focused on degradation of malonyl ginsenosides and transformation into less polar and rare ginsenosides. Changes in the neutral and malonyl ginsenosides were only determined in American ginseng powder and the storage time was short.

In this study, effects of malonyl ginsenosides on evaluation and quality control of *P. ginseng* roots was investigated. The content of neutral and malonyl ginsenosides were quantified by high-performance liquid chromatography equipped with UV-VIS detector (HPLC-UV) during storage and extraction of different ginseng products.

2. Experimental

2.1. Materials

Ginsenoside Rb_1, Rb_2, Rc, Rd, Re and Rg_1 were obtained from College of Fundamental Medical, Jilin University (Changchun, China). Malonyl ginsenoside Rb_1, Rb_2, Rc, Rd and ginsenoside Ro were isolated and purified from fresh *P. ginseng* in our laboratory, and identified by IR, LCQ-MS, ^{1}H NMR and ^{13}C NMR spectroscopic data [13,14]. The fresh *P. ginseng* roots were harvested in Jilin Province of China in 2009. The ginseng root was rinsed with water, dried at 30 °C, powdered, and passed in a 40 mesh sieve. Acetonitrile and MeOH were HPLC grade (Fisher Scientific, USA). Analytical grade methanol, ethanol and *n*-butanol were obtained from Beijing Chemical Reagent Factory. Water was purified by Milli-Q system (Millipore, Bedford, MA, USA). All solutions were filtered through a 0.45 μm hydrophilic polypropylene membrane before use.

2.2. Quantitative analysis method

An Agilent 1100 liquid chromatography (Agilent Technologies, USA) equipped with quaternary gradient pump and a UV detector was used. The mixture of standard ginsenosides or the samples of extraction were dissolved in 25 ml of methanol and filtered through filters [0.45 μm (Millipore)] for HPLC analysis. The analytical column was a Cosmosil C18, 5 μm, 250 mm × 4.6 mm. The separation of ginsenosides was obtained by gradient elution using acetonitrile (A) and 0.05 M KH_2PO_4 (B) as the eluents. The gradient profile was: 0–22 min, 21% (A), 79% (B); 22–30 min, 21–29% (A), 79–71% (B); 30–50 min, 29% (A), 71% (B); 50–60 min, 29–35% (A), 71–65% (B); 60–70 min, 35–50% (A), 65–50% (B). The flow rate was kept at 1.0 ml/min. Injection volume was 20 μl. The chromatogram was monitored at 203 nm.

2.3. Extraction methods

2.3.1. Cold-soaked extraction (CSE)

A sample of *P. ginseng* root 1 g was placed into a 100 ml conical flask, and extracted with 50 ml of 70% (v/v) methanol–water at room temperature (25 °C) five times, every time for 12 h. When the extraction was completed, the extract was filtrated and transferred into a 500 ml flask, and the conical flask was rinsed three times with the extraction solvent. The solvent used for rinsing was also added to the flask. The extract was concentrated under reduced pressure at 40 °C, and the residue was dissolved in 25 ml in methanol, and then filtered for analysis.

2.3.2. Soxhlet extraction (SE)

A 1 g sample of *P. ginseng* root was accurately weighed and placed into a 250 ml flask of a Soxhlet extractor. One hundred milliliters of 70% (v/v) methanol solution was added into it. The mixture was refluxed in a water bath (85 °C) for 24 h to extract the ginsenosides. When the extraction was completed, the procedure was followed as described in Section 2.3.1.

2.3.3. Heat reflux extraction (HRE)

1 g of *P. ginseng* root was mixed with 50 ml of 70% (v/v) methanol–water in a 200 ml round bottom flask fitted with a cooling condenser. The flask was heated in 80 °C water bath three times and every time for 2 h. When the extraction was completed, the procedure was followed as described in Section 2.3.1.

2.3.4. Microwave-assisted extraction (MAE)

A 1 g sample of *P. ginseng* root was put into a 150 ml conical flask. After adding 50 ml of 70% methanol–water (v/v), the flask was exposed to the microwave. The microwave extractor was operated at 200 W with an emission frequency of 2450 MHz under atmospheric pressure condition and the extraction was carried out three times and every time for 5 min. When the extraction was completed, the procedure was followed as described in Section 2.3.1.

2.3.5. Ultrasound-assisted extraction (UAE)

A sample of *P. ginseng* root 1 g was placed into a 100 ml conical flask, in which 50 ml of 70% (v/v) methanol–water was added. Then the flask was sonicated three times and every time for 20 min in an ultrasonic water bath. Ultrasound-assisted extraction was performed with a KQ2200E Ultrasonic Cleaners (Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China). The output power was 250 W and the frequency was 50 kHz. When the extraction was completed, the procedure was followed as described in Section 2.3.1.

2.4. Ginseng treatments and storage

Freshly harvested *P. ginseng* roots, grown in Jilin Province of China, were used for all experiments. In each study, all roots were of a uniform size and appearance. The fresh *P. ginseng* roots were washed, then separated into three groups and the weight of each group was determined. White ginseng is produced by a hot air drier and ventilated with air at 38 °C. Red ginseng was manufactured by steaming fresh ginseng at 95–100 °C for 2–3 h. After the processing of white ginseng and red ginseng were terminated, the water content remaining in the roots was ~10%, similar to the level found in commercially dried roots. The white ginseng was then stored in a

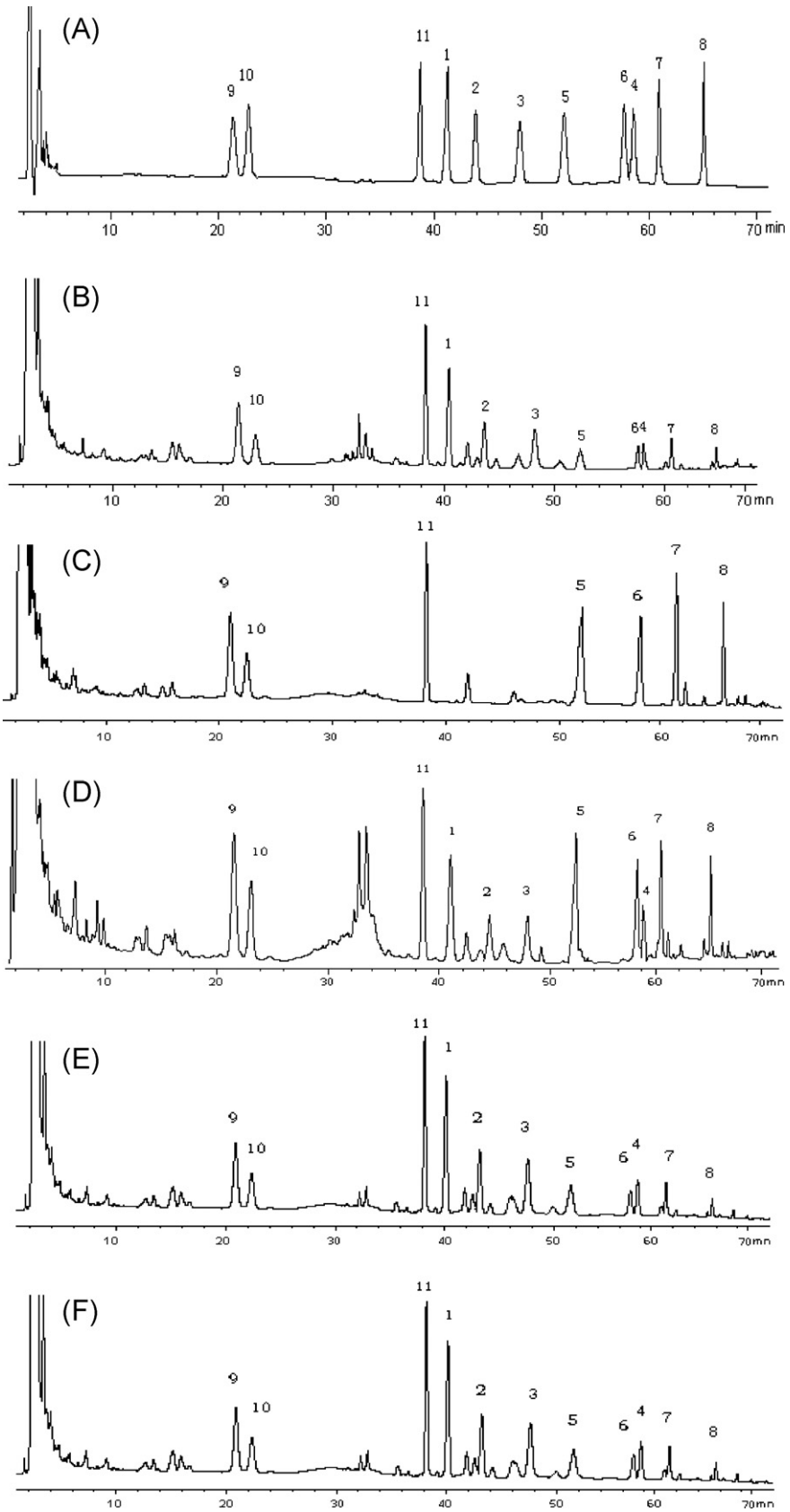


Fig. 2. HPLC chromatograms of mixed standards (A), cold-soaked extraction (B), Soxhlet extraction (C), heat reflux extraction (D), microwave-assisted extraction (E) and ultrasonic-assisted extraction (F). Peak 1: malonyl-ginsenoside Rb_1; peak 2: malonyl-ginsenoside Rc; peak 3: malonyl-ginsenoside Rb_2; peak 4: malonyl-ginsenoside Rd; peak 5: ginsenoside Rb_1; peak 6: ginsenoside Rc; peak 7: ginsenoside Rb_2; peak 8: ginsenoside Rd; peak 9: ginsenoside Rg_1; peak 10: ginsenoside Re; peak 11: ginsenoside Ro.

Table 1
Calibration curve and concentration range of 11 ginsenosides.

Ginsenoside	Calibration curve[a]	Correlation coefficient (r^2)	Test range (μg/ml)
M-Rb_1	$y = 2241.2x - 2.79$	0.9997	10.12–647.68
M-Rb_2	$y = 2188.7x - 3.42$	0.9996	9.97–638.08
M-Rc	$y = 2272.2x - 5.24$	0.9996	10.11–647.04
M-Rd	$y = 2125.0x - 7.01$	0.9991	10.03–641.92
Rb_1	$y = 2430.7x - 3.56$	0.9998	10.08–645.12
Rb_2	$y = 1991.4x - 2.86$	0.9995	10.10–646.40
Rc	$y = 2743.6x - 8.64$	0.9987	10.20–652.80
Rd	$y = 2322.8x - 3.25$	0.9999	9.97–638.08
Re	$y = 2171.5x - 2.01$	0.9992	10.11–647.04
Rg_1	$y = 2272.3x - 3.48$	0.9993	9.95–636.80
Ro	$y = 2395.2x - 5.52$	0.9990	10.01–640.60

[a] y, peak area; x, concentration (mg/ml).

room held at 25 °C in air of <15% relative humidity. Analyses were conducted after storage for 0, 3, 6, and 9 months.

2.5. Conventional heat treatment and alkaline hydrolysis

The mixed standard containing 10 mg of each malonyl ginsenoside (M-Rb_1, M-Rc, M-Rb_2 and M-Rd) was dissolved in 70% methanol (10 ml). The degradation of malonyl ginsenoside was performed with microwave heating for 5 min, ultrasonic water bath for 20 min at room temperature, water bath for 24 h at 85 °C and 5% KOH solution (1 ml) for 2 h at room temperature [17,35], respectively.

3. Results and discussion

3.1. Determination of ginsenosides

HPLC-UV was used to analyze the ginsenoside. The HPLC conditions were optimized by changing the elution gradient. Malonyl ginsenoside Rb_1, Rc, Rb_2, Rd, ginsenoside Ro, Rg_1, Re, Rb_1, Rc, Rb_2 and Rd in the extract of sample were identified by comparison of the retention times with those obtained from chromatograms of the mixed ginsenoside standards. The chromatograms are shown in Fig. 2. The chromatographic separation of the malonyl ginsenosides and the neutral ginsenosides in a single run was achieved by linear gradient elution. Under these HPLC conditions, each ginsenoside was well resolved, hence this method can be used for the quantitative determination of ginsenosides in ginseng sample. Compared to the HPLC method described by others [15–17], KH_2PO_4 was necessary for the simultaneous quantification of neutral and malonyl ginsenosides. Owing to the acidity of the malonyl ginsenosides, a phosphate buffer was used in the mobile phase for elution.

The linearity of the calibration curves was verified by the correlation coefficients and the results are shown in Table 1. The precision of the HPLC method was determined by intra- and inter-day variations. A sample of 1 g *P. ginseng* root was analyzed and extracted as described in Sections 2.2 and 2.3.1. The intra-day precision was finished in one day and three parallel experiments were carried out. The inter-day precision was studied by performing the same process in three different days. The intra-day and inter-day precision (repeatability) was 1.02–2.88% and 2.24–4.55%, respectively. The recoveries of ginsenosides were determined with spiked samples. Accurate amounts of 11 ginsenosides were added to approximately 1 g of *P. ginseng* root and then extracted and analyzed as described in Sections 2.2 and 2.3.1. The recoveries of all 11 ginsenosides were within the range of 97.4–103.6% ($n = 6$).

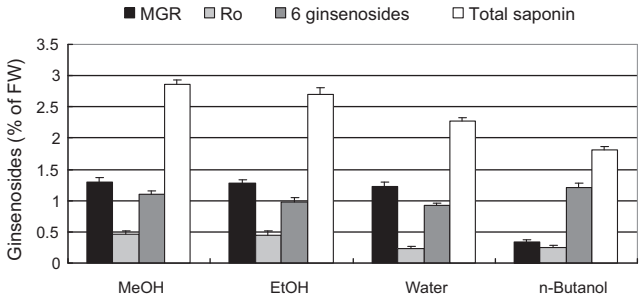


Fig. 3. Extraction of ginsenoside by different solvents.

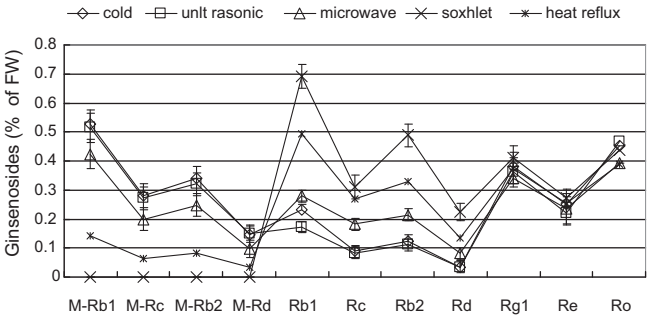


Fig. 4. Individual ginsenoside change by different methods.

3.2. The effect of solvent

Several solvents were used to extract ginsenoside from *P. ginseng* root, such as water, ethanol–water solution, methanol–water solution, and water–saturated *n*-butanol. Due to the difference of polarities of these four extraction solvents, the solubility of ginsenosides and the rate of mass transfer were different. The yields of the ginsenosides extracted by CSE with different extraction solvents are shown in Fig. 3. The extraction yields of ginsenosides in methanol–water solution are the highest and the extraction yields in *n*-butanol are the lowest. As a result, the optimal extraction solvent is methanol–water for CSE.

3.3. Comparison of different extraction methods

To investigate the effect of the extraction method, the yield obtained by CSE was compared with other extraction methods, SE, UAE, MAE, and HRE using 70% (v/v) methanol–water as the solvent for extraction of six neutral ginsenosides and four malonyl ginsenosides from *P. ginseng* root. Figs. 4 and 5 show the extraction yields of ginsenosides, especially malonyl ginsenoside Rb_1, Rc, Rb_2 and Rd. The yields obtained by CSE for 12 h and UAE for 20 min were much higher than those by MAE for 5 min and HRE for 2 h, but ginsenosides were hardly found in the extract obtained by SE for 24 h. The

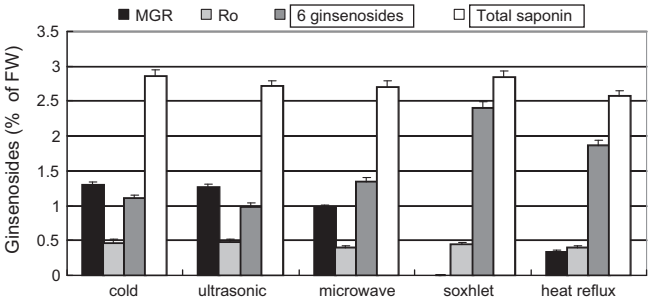


Fig. 5. Extraction of ginsenosides by different methods.

Table 2
Contents of ginsenosides (mg/g, $n = 3$) in fresh ginseng, red ginseng, and suncured ginseng.

Ginsenoside	Fresh ginseng	Red ginseng	Suncured ginseng during storage			
			0 month	3 months	6 months	9 months
M-Rb_1	5.27 ± 0.08	0	5.13 ± 0.05	4.27 ± 0.07	3.33 ± 0.06	2.92 ± 0.05
M-Rc	2.82 ± 0.05	0	2.65 ± 0.02	2.09 ± 0.03	1.65 ± 0.02	1.34 ± 0.04
M-Rb_2	3.41 ± 0.03	0	2.94 ± 0.02	2.31 ± 0.06	1.94 ± 0.05	1.59 ± 0.03
M-Rd	1.44 ± 0.09	0	1.15 ± 0.07	1.02 ± 0.06	0.85 ± 0.04	0.43 ± 0.05
Rb_1	2.31 ± 0.07	4.26 ± 0.04	1.88 ± 0.06	2.47 ± 0.05	3.18 ± 0.06	3.72 ± 0.08
Rc	0.88 ± 0.07	3.19 ± 0.06	0.98 ± 0.04	1.22 ± 0.03	1.68 ± 0.04	1.87 ± 0.06
Rb_2	1.24 ± 0.06	3.15 ± 0.07	1.36 ± 0.03	1.85 ± 0.02	2.06 ± 0.06	2.45 ± 0.04
Rd	0.35 ± 0.05	1.83 ± 0.03	0.58 ± 0.06	0.884 ± 0.07	1.18 ± 0.05	1.22 ± 0.08
Rg_1	3.81 ± 0.08	2.85 ± 0.05	3.74 ± 0.09	3.64 ± 0.10	3.50 ± 0.07	3.52 ± 0.06
Re	2.49 ± 0.04	1.86 ± 0.08	2.63 ± 0.07	2.58 ± 0.03	2.46 ± 0.09	2.49 ± 0.02
Ro	4.54 ± 0.06	3.95 ± 0.03	4.60 ± 0.08	4.37 ± 0.05	4.13 ± 0.06	4.26 ± 0.07
Total	28.56 ± 0.26	21.09 ± 0.10	27.64 ± 0.17	26.70 ± 0.18	25.96 ± 0.15	25.81 ± 0.14

degradation of malonyl ginsenosides depends on the temperature and extraction time.

3.4. Effect of processing and storage

The data in Table 2 show the changes in the concentrations of neutral, malonyl and total ginsenosides in *P. ginseng* root extracts by CSE during storage at 0, 3, 6 and 9 months at 25 °C in air of <15% relative humidity. The concentration of malonyl ginsenosides in white ginseng showed a highly significant decrease and the corresponding neutral ginsenosides a significant increase during storage from 0 to 9 months, while the concentration of total ginsenosides was not significantly changed. The content of ginsenosides was lower in red ginseng than in fresh ginseng and white ginseng, and no MGR could be detected. Malonyl ginsenoside was transformed into corresponding neutral ginsenoside and less polar and rare ginsenosides during the processing of red ginseng [29].

3.5. Degradation of malonyl ginsenoside

Degradation of malonyl ginsenosides did not occur in UAE for 20 min at room temperature. Malonyl ginsenosides exist in MAE, but can be hardly found in water bath at 85 °C and in 5% KOH solution. Fig. 6A and B shows that malonyl ginsenosides transformed into corresponding neutral ginsenoside and did not degrade into less polar and rare ginsenosides in water bath at 85 °C.

Malonyl ginsenosides, which are thermally unstable, can degrade into corresponding neutral ginsenosides [30,31]. Our results show that the neutral and malonyl ginsenosides make up to 85–90% of total content in ginsenosides. The content of malonyl ginsenosides is two-fold higher than neutral ginsenoside in the fresh ginseng, about 45% of total ginsenosides. Awang et al. also reported that steaming the North American ginseng converts the malonyl ginsenosides to their corresponding, demalonylated, neutral ginsenosides, which can result in as much as a two-fold increase in Rb_1 content [22].

The findings from this study demonstrated the importance of analyzing both neutral and malonyl ginsenosides to determine the true ginsenosides content of a ginseng product. It showed that only using neutral ginsenoside as the main index will underestimate and confuse the ginseng product evaluation and quality control. This was illustrated in the processing and storage trial, whereas the content of malonyl ginsenosides from white ginseng decreased obviously with increasing storage time. Malonyl ginsenosides are in dynamic state in *P. ginseng* during processing and storage. The content of total saponins including neutral and malonyl ginsenosides have no significant difference. Therefore, the degradation of malonyl ginsenosides induces confusion for evaluation and quality control of ginseng product.

Neutral ginsenosides analysis can give a misleading impression of the efficacy of an extraction method. This was illustrated in the extraction trial, where the content of malonyl ginsenosides in the same white ginseng ranged from 0% to 1.35% with using different extraction methods, whereas this was undoubtedly due to hydrolysis of malonyl ginsenosides to neutral ginsenosides so that the total ginsenosides were actually increasing as the extraction temperature and time increased. Wang et al. reported that malonyl ginsenosides exist in the extract obtained by Soxhlet extraction for 2 h [6]. Court et al. showed that, while partial degradation (50%) occurred after 5 h of extraction using methanol in Soxhlet apparatus, a minimum of 20 h was necessary to achieve total conversion [17]. In our work, malonyl ginsenosides cannot be found after Soxhlet extraction for 24 h. The malonyl ginsenosides transformed into corresponding neutral ginsenoside and did not degrade into less polar and rare ginsenosides with Soxhlet method. Measurement of malonyl ginsenosides was also evaluated by the different solvents, including water, ethanol, methanol, and *n*-butanol. The optimum extraction solvent was obtained with 70% methanol. The *n*-butanol was found to be the most efficient solvent for the neutral ginsenosides, but the extraction yields for malonyl ginsenosides was the lowest.

The compound at Rt 38.5 min has been identified as ginsenoside Ro by comparing retention times with authentic compound. In our study, we have found ginsenoside Ro was relatively stable during processing, storage and extraction (Table 2 and Figs. 3–5). Ginsenoside Ro is more difficult to analyze by HPLC than the neutral ginsenosides without using a phosphate buffer in the mobile phases [11,21]. This suggested that ginsenoside Ro is not suitable as index for ginseng product evaluation and quality control. Although the content of total ginsenosides was also underestimated by ignoring ginsenoside Ro, it cannot induce the confusion for evaluation and quality control of ginseng product.

The variability of cultivation conditions (e.g., soil, temperature, moisture, length of cultivation, and harvest season) affects the total ginsenoside concentration and the percentages of individual ginsenosides in *P. ginseng*. Therefore, the total content of ginsenosides in *P. ginseng* was significantly different during processing, storage and extraction, which should be ascribed to dynamic changes of malonyl ginsenosides.

Evaluation and quality control of Chinese medicines include qualitative and quantitative analysis, while quantitative determination is usually not available without reference compound as standard. Especially, some pure chemical compounds are difficult to obtain or store because of their instability and/or trace amount [32]. Malonyl ginsenosides are more unstable and not easily available than the neutral ginsenosides as standard for the HPLC determination. Therefore, the contents of neutral ginsenosides have been developed for quality control and evaluation of

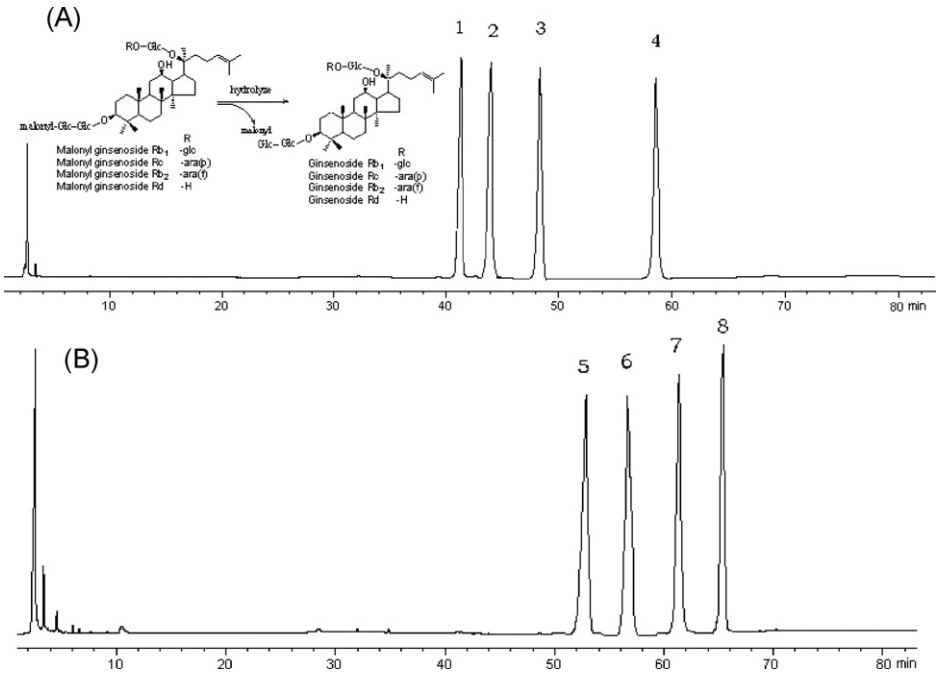


Fig. 6. HPLC chromatograms of mixed standard malonyl ginsenosides (A) and degradation of malonyl ginsenosides in 70% methanol solution under water bath for 24 h at 85 °C (B). Peak 1: malonyl-ginsenoside Rb_1; peak 2: malonyl-ginsenoside Rc; peak 3: malonyl-ginsenoside Rb_2; peak 4: malonyl-ginsenoside Rd; peak 5: ginsenoside Rb_1; peak 6: ginsenoside Rc; peak 7: ginsenoside Rb_2; peak 8: ginsenoside Rd.

P. ginseng by any trained HPLC operator [33]. For example, the American Botanical Council started the Ginseng Evaluation Program (GEP) in 1993 [34]. The part one of GEP presented data on the evaluation of consistency of standardized ginseng products through the HPLC-UV quantification of ginsenosides Rb_1, Rb_2, Rc, Re, Rg_1 and Rd.

Sample preparation is one of the key steps which greatly influences the repeatability and accuracy of quantitative analysis. It is reported that more than 60% of analysis error derived from non-standard sample pretreatment [32]. Up to now, many conventional extraction methods have been employed for the extraction of ginsenosides from ginseng products including CSE, SE, HRE, UAE, MAE, etc. High pressure microwave assisted extraction (HPMAE) and ultrahigh pressure extraction (UPE) are relatively new extraction methods. Compared with these new extraction techniques, the conventional extraction methods are more widely used for the ginseng product evaluation and quality control. It has become an important task to establish an authoritative evaluation and quality control standard for ginseng products. However, sample preparation of ginseng products has not been extensively studied and ignored malonyl ginsenosides. According to our results, SE could be directly used as an evaluation methodology of total ginsenosides with achieving total malonyl ginsenosides conversion after extraction and in the other extraction methods malonyl ginsenosides should be hydrolyzed by alkaline hydrolysis to convert to their corresponding neutral ginsenosides. The hydrolysis was performed by removing the methanol in original extract then mixing the residue with 5% potassium hydroxide solution. The hydrolyzed solution was neutralized with potassium hydrogen phosphate and made up to volume with acetonitrile [17,35].

4. Conclusions

The effect of malonyl ginsenosides on evaluation and quality control of *P. ginseng* roots was investigated. The content of neutral and malonyl ginsenosides in different ginseng products was determined by HPLC-UV and the results obtained by different extraction methods were compared. The content of malonyl ginsenosides from white ginseng decreased with increasing storage time. The content of malonyl ginsenosides in the same white ginseng ranged from 0% to 1.35% using different extraction methods, while the content of total saponins including neutral and malonyl ginsenosides remained constant. The total ginsenosides was not only underestimated but also determined imprecisely by ignoring malonyl ginsenosides. We suggest that malonyl ginsenosides should be transformed into the corresponding neutral ginsenosides during sample preparation for quality control and evaluation of *P. ginseng*. Then the content of six neutral ginsenosides in samples can be used as the true level of total ginsenosides.

Acknowledgment

This work was supported Jilin Science & Technology Development Plan (20100914).

References

[1] K.Y. Lee, Y.H. Lee, S.I. Kim, J.H. Park, S.K. Lee, Ginsenoside-Rg_5 suppresses cyclin E-dependent protein kinase activity via up-regulation of p21 Waf1 with concomitant down-regulation of cdc25A in SK-HEP-1 cells, Anticancer Res. 17 (1997) 1067–1072.
[2] Y.W. Shin, E.A. Bae, D.H. Kim, Inhibitory effect of ginsenoside Rg_5 and its metabolite ginsenoside Rh_3 in an oxazolone-induced mouse chronic dermatitis model, Arch. Pharm. Res. 29 (2006) 685–690.
[3] A.S. Attele, Y.P. Zhou, J.T. Xie, J.A. Wu, L. Zhang, L. Dey, W. Pugh, P.A. Rue, K.S. Polonsky, C.S. Yuan, Antidiabetic effects of *Panax ginseng* berry extract and the identification of an effective component, Diabetes 51 (2002) 1851–1858.
[4] B.H. Lee, J.H. Lee, I.S. Yoon, J.H. Lee, S.H. Choi, T.J. Shin, M.K. Pyo, W.S. Choi, S.M. Lee, Y. Lim, H. Rhim, S.Y. Nah, Mutations of arginine 222 in pre-transmembrane domain I of mouse 5-HT3A receptor abolish 20(R)-but not 20 (S)-ginsenoside Rg_3 inhibition of 5-HT-mediated ion currents, Biol. Pharm. Bull. 30 (2007) 1721–1726.
[5] X.H. Shen, Y.P. Ren, Y. Chen, Detection of ginsenoside in healthy foods by HPLC, Chin. J. Health Lab. Technol. 5 (2003) 600–601.
[6] Y.T. Wang, J.Y. You, Y. Yu, C.L. Qu, H.R. Zhang, L. Ding, H.Q. Zhang, X.W. Li, Analysis of ginsenosides in *Panax ginseng* in high pressure microwave-assisted extraction, Food Chem. 110 (2008) 161–167.
[7] W. Shi, Y.T. Wang, X.J. Quan, L.F. Bai, H.R. Zhang, X.D. Chen, L. Ding, H.Q. Zhang, Determination of ginsenosides in the root of radix ginseng by high

performance liquid chromatography/evaporative light scattering detection, Chin. J. Anal. Chem. 34 (2006) 243–246.
[8] X.W. Du, R.B.H. Wills, D.L. Stuart, Changes in neutral and malonyl ginsenosides in American ginseng (*Panax quinquefolium*) during drying, storage and ethanolic extraction, Food Chem. 86 (2004) 155–159.
[9] N. Fuzzati, B. Gabetta, K. Jagaker, R. Pace, F. Peterlongo, Liquid chromatography–electrospray mass spectrometric identification of ginsenosides in *Panax ginseng* roots, J. Chromatogr. A 854 (1999) 69–79.
[10] A.L. Lau, S.O. Woo, H.L. Koh, Analysis of saponins in raw and steamed *Panax notoginseng* using high-performance liquid chromatography with diode array detection, J. Chromatogr. A 1011 (2003) 77–87.
[11] G.X. Ren, F. Chen, Degradation of ginsenosides in American ginseng (*Panax quinquefolium*) extracts during microwave and conventional heating, J. Agric. Food Chem. 47 (1999) 1501–1505.
[12] H.J. Zhang, Y.J. Wu, Y.Y. Cheng, Analysis of 'SHENMAI' injection by HPLC/MS/MS, J. Pharm. Biomed. Anal. 31 (2003) 175–183.
[13] G.Z. Sun, Z. Liu, X.G. Li, Y.N. Zheng, J.Y. Wang, Isolation and identification of two malonyl-ginsenosides from the fresh root of *Panax ginseng*, Chin. J. Anal. Chem. 33 (2005) 1783–1786.
[14] G.Z. Sun, X.G. Li, Z. Liu, J.Y. Wang, Y.N. Zheng, X.W. Yang, Isolation and structure characterization of malonyl-notoginsenoside-R_4 from the root of *Panax ginseng*, Chem. J. Chin. Univ. 28 (2007) 1316–1318.
[15] G.X. Ren, F. Chen, Simultaneous quantification of ginsenosides in American ginseng (*Panax quinquefolium*) root powder by visible/near-infrared reflectance spectroscopy, J. Agric. Food Chem. 47 (1999) 2771–2775.
[16] G.C. Kite, M.J.R. Howes, C.J. Leon, M.S.J. Simmonds, Liquid chromatography/mass spectrometry of malonyl-ginsenosides in the authentication of ginseng, Mass Spectrom. 17 (2003) 238–244.
[17] W.A. Court, J.G. Hendel, J. Elmi, Reversed-phase high performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*, J. Chromatogr. A 755 (1996) 11–17.
[18] M.R. Harkey, G.L. Henderson, M.E. Gershwin, J.S. Stern, R.M. Hackman, Variability in commercial ginseng products: an analysis of 25 preparations, Am. J. Clin. Nutr. 73 (2001) 1101–1106.
[19] N. Fuzzati, Analysis methods of ginsenosides, J. Chromatogr. B 812 (2004) 119–133.
[20] W. Lim, K.W. Mudge, F. Vermeylen, Effects of population, age, and cultivation methods on ginsenoside content of wild American ginseng (*Panax quinquefolium*), J. Agric. Food Chem. 53 (2005) 8498–8505.
[21] W.C. Chuang, H.K. Wu, S. Sheu, S.H. Chiou, H.C. Chang, Y.P. Chen, A comparative study on commercial samples of ginseng radix, Planta Med. 61 (1995) 459–465.
[22] D.V.C. Awang, The neglected ginsenosides of North American ginseng (*Panax quinquefolius* L.), J. Herbs Spices Med. Plants 7 (2000) 103–109.
[23] R.M. Corbit, J.F.S. Ferreira, S.D. Ebbs, L.L. Murphy, Simplified extraction of ginsenosides from American ginseng (*Panax quinquefolius* L.) for high-performance liquid chromatography-ultraviolet analysis, J. Agric. Food Chem. 53 (2005) 9867–9873.
[24] S. Gafner, C. Bergeron, M.M. Mccollom, L.M. Cooper, K.L. Mcphall, W.H. Gerwick, Evaluation of the efficiency of three different solvent systems to extract triterpene saponins from roots of *Panax quinquefolius* using high-performance liquid chromatography, J. Agric. Food Chem. 52 (2004) 1546–1550.
[25] B. Vongsangnak, J. Gna, S. Chauvatcharin, J.J. Zhong, Towards efficient extraction of notoginseng saponins from cultured cells of *Panax notoginseng*, Biochem. Eng. J. 18 (2004) 115–120.
[26] H.C. Wang, C.R. Chen, C.J. Chang, Carbon dioxide extraction of ginseng root hair oil and ginsenosides, Food Chem. 72 (2001) 505–509.
[27] S.Q. Zhang, R.Z. Chen, H. Wu, C.Z. Wang, Ginsenoside extraction from *Panax quinquefolium* L. (American ginseng) root by using ultrahigh pressure, J. Pharm. Biomed. Anal. 41 (2006) 57–63.
[28] S.N. Kim, Y.W. Ha, H. Shin, S.H. Son, S.J. Wu, Y.S. Kim, 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 (2007) 164–170.
[29] X.G. Li, L. Fu, Q. Lu, X. Li, Study on hydrolysis reaction of ginsenoside and products in red ginseng processing, J. Jilin Agric. Univ. 22 (2000) 1–9.
[30] I. Kitagawa, T. Taniyama, T. Hayashi, M. Yoshikawa, Malonyl-ginsenoside Rb_1, Rb_2, Rc, Rd, four new malonylated dammarane-type triterpene oligoglucoside from ginseng radix, Chem. Pharm. Bull. 31 (1983) 3353–3356.
[31] C.C. Ruan, Z. Liu, X. Li, X. Liu, L.J. Wang, H.Y. Pan, Y.N. Zheng, G.Z. Sun, Y.S. Zhang, L.X. Zhang, Isolation and characterization of a new ginsenoside from the fresh root of *Panax ginseng*, Molecules 15 (2010) 2319–2325.
[32] S.P. Li, J. Zhao, B. Yang, Strategies for quality control of Chinese medicines, J. Pharm. Biomed. Anal. 55 (2011) 802–809.
[33] J.H. Chen, M.Y. Xie, Z.H. Fu, F.S.C. Lee, X.R. Wang, Development of a quality evaluation system for *Panax quinquefolium* L. based on HPLC chromatographic fingerprinting of seven major ginsenosides, Microchem. J. 85 (2007) 201–208.
[34] T. Hall, Z. Lu, P.N. Yat, J.F. Fitzloff, J.T. Arnason, D.V.C. Awang, H.H.S. Fong, M. Blumenthal, Evaluation of consistency of standardized Asian ginseng products in the ginseng evaluation program, HerbalGram 52 (2001) 31–45.
[35] I. Kitagawa, I. Taniyama, M. Yoshikawa, Y. Ikenishi, Y. Nakagawa, Chemical studies on crude drug processing VI. Chemical structures of malonyl-ginsenosides Rb_1, Rb_2, Rc, and Rd isolated from the root of *Panax ginseng* C.A. Meyer, Chem. Pharm. Bull. 37 (1989) 2961–2970.