Comparative Titration of Ginsenosides by Different Techniques in Commercial Ginseng Products and Callus Cultures

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Abstract

The ginsenoside content of different ginseng species (*Panax ginseng, P. quinquefolium*, and *P. vietnamensis*) from different sources (roots from field-grown plants or from in vitro cultures, cells from solid calluses or from liquid cultures, commercial powders, and suspensions) is evaluated by means of a new high-performance thin-layer chromatography (HPTLC) technique combining an automatic TLC sampler and scanner. The results are compared with those obtained through more classical gross spectrometric and high-performance liquid chromatography (HPLC) techniques. HPTLC and HPLC allow the separation and estimation of the different ginsenosides. For this, HPTLC is faster and simpler than HPLC. Both techniques determine less amounts of ginsenosides than spectrophotometry, which displays overestimated values caused by light absorption by contaminating osides. In vitro cultured cells and roots contain the same ginsenosides as those produced by their mother plants, although at quite lower levels. The culture media also accumulates ginsenosides.

Introduction

Panax ginseng (PG) CA Meyer (ginseng) and its two congeners, *P. notoginseng* (Burk) F.H. Chen (Sanchi ginseng) and *P. quinquefolium* L. (American ginseng), are plants belonging to the Araliaceae family, and their roots are well-known plant drugs that have traditionally been used as a vitalizing and stimulating agent since ancient times. Formerly used especially in Asia and North America, ginseng roots are now widely consumed in many countries of the world (1). Ginseng is very expensive because of its slow growth (5–7 years) and troublesome cultivation (2). For this reason, in vitro cultured ginseng tissues may provide an alternative supply of fresh raw materials.

The main active substances of *P. ginseng* are ginsenosides (triterpenic dammaranic saponins). More than 30 different ginsenosides are known; pharmaceutical experts consider Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 to be the most representative. The quantitative and qualitative ginsenoside compositions of the preparations are particularly important because ginsenosides of groups Rb and Rg have different pharmacological properties, some of them being antagonistic, hyper-, or hypo-tensors for instance (3). It is therefore very important to know the exact ginseng material composition in order to determine its medicinal activity.

In view of the high prices of these products, there is furthermore a need for regulations regarding the control of commercial ginseng products. This is more urgent because raw materials are becoming available from different biotechnological procedures including callus and cell cultures (4–7), hairy root cultures (8–10), and somatic embryos (11–15).

Another problem is that most product labels—when they are labelled—provide the ginsenoside levels on the basis of a global assay by a spectrophotometric determination of crude extracts according to the German pharmacopoeia, Deutsches Arzneibuch (DAB) 10 (16). This type of titration does not take into account possible interferences with other products. The aim of the present work was precisely to compare the results of ginsenoside titration by a new high-performance thin-layer chromatography (HPTLC) technique in commercial ginseng products and callus, cell, and root cultures, with the results obtained with other techniques already mentioned in literature (spectrophotometry, cumulative high-performance liquid chromatography (HPLC)] (16,17).

Experimental

Callus and cell cultures

One-year-old roots of PG CA Meyer (from a Belgian field culture) and of *P. quinquefolium* (ORTIS Laboratories, Elsenborn, Belgium) were surface-sterilized by successive incubations in ethanol (70%) for 3 min and sodium hypochlorite (3%) for 20 min. The roots were rinsed three times with sterile distilled water.

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Calluses were initiated from 3-mm root sections that were cultured on a solid Murashige and Skoog (MS) basal medium (18) supplemented with 0.3 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L of kinetin (KIN). The cultures were incubated in darkness at 25°C ± 2°C in 9-cm diameter petri dishes for 6 weeks. The callus was subcultured every 4 weeks on a solid MS basal medium, supplemented with 2,4-D (1 mg/L) and KIN (0.1 mg/L) in darkness. Callus of *P. vietnamensis* was kindly provided by M.R. Thils of the society Plant 2000 (Brussels, Belgium).

Liquid cell cultures

The cultures were initiated by transferring 2.5 g of finely minced callus to 100 mL conical flasks containing 50 mL of half strength MS (MS/2) medium supplemented with auxin (1 mg/L of 2,4-D) and cytokinin (0.1 mg/L of KIN) for 1 month with shaking (80 rpm) at 25°C ± 2°C in darkness (19). Such cultures were treated (elicited) for 9 days by potential elicitors of ginsenoside production when aged after 3 weeks.

Hairy-like roots

The hairy-like roots of PG CA Meyer were produced as previously described in the literature (9).

The standard ginsenosides

Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 were purchased from Extrasynthèse (Lyon, France).

Ginsenoside extraction

Method 1 was according to the German pharmacopoeia, DAB 10 (16). Lyophilized powdered samples (1 g) were extracted in 70 mL 50% methanol with 0.15% polydimethylsiloxan R for 1 h at 100°C and centrifuged 15 min at 15,000 g. After evaporation of the supernatant, the residue was dissolved in 20 mL of 0.1M HCl. The solution was successively extracted three times by 70 mL of the upper phase of the solution chloroform–0.1M HCl–1-butanol (30:90:180). The three upper phases were added and washed two times with 30 mL of the lower phase of the same solution. After evaporation of the 2 pooled upper phases, the residue was dissolved in 50 mL 98% acetic acid. One milliliter of this solution was added to 4 mL of the mixture 98% acetic acid–sulphuric acid (50:50, v/v) and incubated 25 min at 60°C. Spectrophotometric determination occurred directly after cooling.

Method 2 was developed in the laboratory. Lyophilized powdered samples (50 mg) were extracted in 10 mL 70% methanol for 1 h at 100°C and centrifuged for 15 min at 16,000 g. After evaporation of the methanolic extract to dryness, the residue was dissolved in 2 mL water and applied to a Isolute C18 EC (500 mg) cartridge and prewashed with 100% methanol (3 mL). After washing the column with water (3 mL) and 50% methanol (3 mL), the ginsenosides were eluted with 4 mL methanol 100%. The solvent was evaporated to dryness and the residue was dissolved in 1 mL of 100% methanol. Culture media, preliminary mixed with pure methanol (1:1), were similarly treated.

Table I. Total Ginsenoside* Titrations in a Raw, Dried, Grinded Powder and in a Commercialized Liquid Suspension† of PG CA Meyer Roots, Following Two Different Extraction Methods

Samples	Extraction method	Determination	Ginsenoside levels
Solid powder	1	Spectrometer	75 ± 8
	1	HPLC	58 ± 4
	2	Spetrometer	88 ± 9
	2	HPLC	78 ± 5
Liquid suspension	1	Spectrometer	8.0 ± 0.9
	1	HPLC	0.6 ± 0.1
	2	Spectrometer	3.8 ± 0.5
	2	HPLC	2.3 ± 0.1

* In mg/g DW.
† Lot number N08A from ORTIS Laboratories.

Ginsenoside determination

Spectrophotometry

Spectrophotometery was carried out according to the German pharmacopoeia, DAB 10 (16). One milliliter of solution was added to 4 mL of a mixture of 98% acetic acid–sulphuric acid (50:50, v/v) and incubated 25 min at 60°C. After cooling, the absorbance at 520 nm was measured. The concentration in ginsenosides [in g/g dry weight (DW)] was determined by the formula 4.24 × optical density. Rb1 ginsenoside was used as the standard to calculate the correlation coefficient.

HPLC

The HPLC method was modified from Schulten and Soldati (17). The HPLC systems consisted of an integrated unit Kontron 32× (Milan, Italy), Hewlett-Packard 1050 autosampler (Palo Alto, CA), UV detector Kontron 432 UV, and Sherwood 386 data processor (Nottingham, U.K.). The peaks were monitored at 203 nm. A stainless steel column (250 × 4 mm) packed with LiChrospher 100 RP 18 was used at 38°C. Samples of 25 µL were applied to the HPLC, and the mobile phase was used in isocratic condition with a flow rate of 1 mL/min.

HPTLC

The systems used consisted of a Camag automatic TLC Sampler 3 and a Camag TLC Scanner 3 (Muttenz, Switzerland). The data were analyzed by the Camag TLC software cats. Five microliters of sample or standard was loaded on HPTLC silica gel 60 13748 plates (Merck, Darmstadt, Germany). The plates were developed in a mixture of chloroform–isobutanol–ethanol–H_2O

Table II. Comparative Titration* of the Total Ginsenoside Content of 3-Year-Old Roots of *Panax quinquefolium*, Crude Grinded Powder of Roots of PG or In Vitro Cultured Roots of PG and PQ by HPLC and HPTLC

	Plant material			
	Powder	Roots	In vitro roots	
Techniques	PG	PQ	PG	PQ
HPLC	77.7 ± 4.8	27.2 ± 2.0	2.9 ± 0.2	0.9 ± 0.1
HPTLC	92.8 ± 6.1	29.7 ± 3.8	3.5 ± 0.5	1.4 ± 0.2

* In mg/g DW.

(20:40:15:20) at room temperature. The peaks were monitored at 203 nm.

The analyses combined the extraction and determination methods. All the results are the means of three different experiments.

Results and Discussion

Typical chromatograms of the ginsenoside standards from the analytical column of the HPLC system look quite similar to those presented by Pietta and Mauri (20), Kanazawa et al. (21), or Chuang and Shen (22), among others.

In a first set of experiments, two different samples of PG, a raw dried grinded powder and a commercialized liquid suspension (ORTIS Laboratories), were analyzed for their ginsenoside contents using spectrophotometric and HPLC methods. Two different techniques of extraction were used. The results are shown in Table I. The extraction method did not influence greatly the results of the HPLC and spectrophotometric determinations for the solid powder, but they did in the case of a commercialized liquid suspension. Method 1, although convenient for the solid powder, did not allow a good extraction of the ginsenosides from the liquid suspension (see HPLC determination) but also entrained other substances contaminating the spectrophotometric titration (see spectrophotometric dosage). The simplest and fastest, method 2, thus appeared the most suitable.

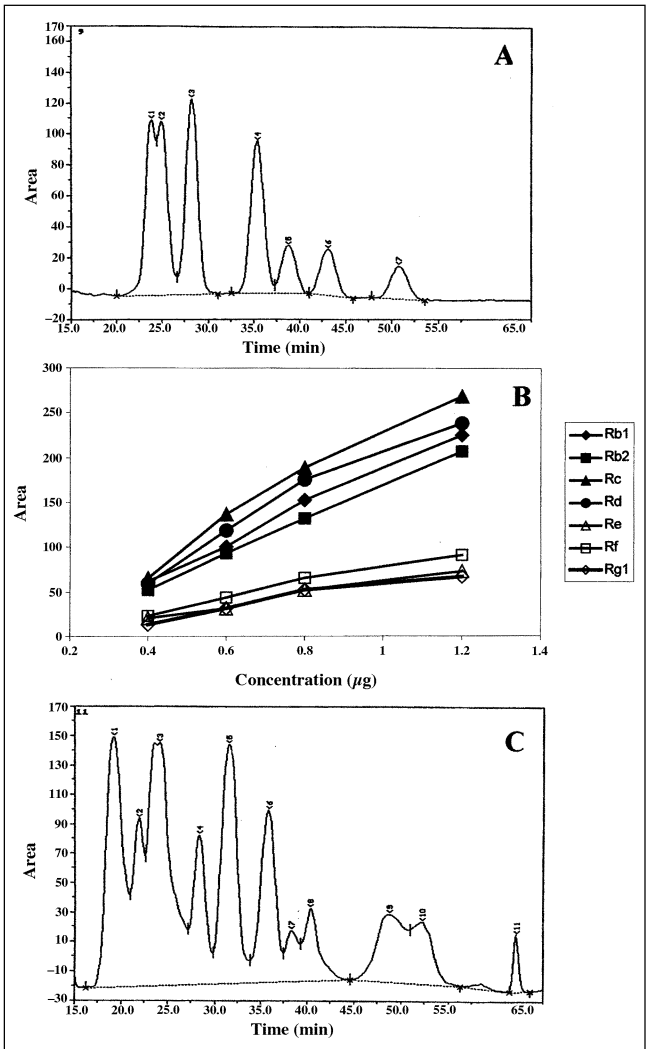

Figure 1. HPTLC chromatograms (fluorescence spectra) of the seven most representative ginsenosides (A), their calibration curves (B), and an extract (70% methanol) of roots of PQ (C).

The amounts determined by spectrophotometry, although the only ones mentioned on the commercialized samples, are still much higher, up to 13 times more. This is attributable to the fact that HPLC sums the seven most representative ginsenosides only but also to the contaminating osides from the crude extracts absorbing at the same wavelength. The spectrophotometric method is thus probably not always adapted for evaluating ginsenoside amounts in the different types of formulations available on the market.

In a second set of experiments, performances of HPLC and HPTLC in titration of ginsenosides were compared. Four different samples were tested: a crude grinded powder of roots of PG, 3-year-old roots of *Panax quinquefolium* (PQ), and in vitro cultured roots of PG and PQ (Table II). Figure 1 presents typical HPTLC chromatograms of the seven most representative ginsenosides (and their calibration curves) and of the 70% methanol extraction of 3-year-old PQ roots.

Results from Table II show that, by two different techniques, the ginsenosides were determined with a higher amount in *P. ginseng* than in *P. quinquefolium* roots (from the nature or from in vitrocultures). Table II also indicates that HPTLC determination generally gives higher amounts of ginsenosides. Notice also the higher amounts of ginsenosides found in field-grown roots than in the in vitro cultured ones.

A detailed analysis of ginsenoside content determined by HPLC and HPTLC in fresh roots of 3-year-old PQ plants and in dried raw grinded powder from PG CA Meyer (ORTIS lot N 07 B, supplier 742.22) is given in Table III. The dried powder contained more sorts of ginsenosides. In addition, quantities of each ginsenoside determined with both analytical techniques are not frequently similar. This reveals the presence of ginsenosides other than the standard ones that interfere with the quantitation of those in a different way for HPLC than for HTPLC. This last technique gives, in all cases, a better quantitation of total ginsenoside content.

In vitro cell and organ cultures are seen as an option for mass production of raw materials. In the case of ginseng, such cultures generally accumulate less ginsenosides than natural organs from field-grown plants (23), which can be compensated by the repetitive mass accumulation in shorter times. Cells are less productive than organs, the secondary metabolites being preferentially accumulated in differentiated (and aged) or regenerating tissues and organs (7,24). This is the reason why investigations are underway to elicit ginsenoside production and accumulation in (more easily) cell cultures (24). Table IV reports the results of such assays of elicitation, using putrescine (PUT), spermidine (Spd), and spermine (Spm) as polyamines and peptone. All of the ginsenoside quantitations were carried out with HPTLC. Notice first the low amounts of ginsenosides accumulated by such cells (in µg/g DW instead of mg/g DW for roots), although of the same kinds as those produced by natural roots. Notice, secondly, that the culture media also accumulated (perhaps secreted) ginsenosides in

Table III. HPLC and HPTLC Determined Ginsenosides and Their Calculated Sum* in 3-Year-Old Roots of PQ and in Crude Grinded Powder of Roots of PG

	Rb1	Rb2	Rc	Rd	Re	Rf	Rg1	Sum
Roots								
HPLC	21.1 ± 1.0	0.9 ± 0.1	1.5 ± 0.3	4.3 ± 0.6	0	0	0	27.8 ± 2.0
HPTLC	15.6 ± 0.8	0	12.0 ± 2.8	2.1 ± 0.2	0	0	0	29.7 ± 3.8
Powder								
HPLC	19.1 ± 0.8	16.0 ± 1.1	12.4 ± 0.7	21.2 ± 1.6	3.9 ± 0.2	0.7 ±0.1	4.4 ± 0.3	77.7 ± 4.8
HPTLC	11.6 ± 0.7	9.4 ± 0.5	11.1 ± 0.6	17.3 ± 1.1	14.8 ± 0.9	14.6 ± 1.3	14.0 ± 1.0	92.8 ± 6.1

* In mg/g DW.

Table IV. Ginsenoside Amounts* in Cells of Calli (PV†, PQ, and PG) and Their Respective Culture Media after 21 or 21+9 Days, the Latter's Being Eventually Supplemented by the Indicated Substances. Determination by HPTLC

	Cells			Media		
	PV	PG	PQ	PV	PG	PQ
Control (21)	2.06	2.70	3.15	1.54	4.27	2.70
Control (21 + 9)	2.71	3.63	4.11	1.53	4.52	3.40
+ PUT 283.6mM	3.68	4.63	5.56	1.92	3.86	3.65
+ SPD 283.6mM	2.74	4.72	6.46	2.55	4.67	2.70
+ SPM 283.6mM	3.46	3.01	3.14	2.47	5.89	7.72
+ Peptone 2g	3.07	3.31	2.35	3.86	6.16	3.52

* In mg/g DW.
† Panax vietnamensis.

amounts quite similar to cells, and, finally, that the polyamines and peptone favored a ginsenoside accumulation, which doubled as compared with the control in certain cases. That ginseng cells released ginsenosides in their culture medium was already known (26).

The beneficial effect of peptone on ginsenoside accumulation had already been shown (25). As far as we know, the elicitation of ginsenoside accumulation by polyamines is shown here for the first time. This effect might be related to the beneficial effect of polyamines for the differentiation of somatic embryos (13).

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References

1. C.X. Liu and P.G. Xiao. Recent advances on ginseng research in China. *J. Ethnopharmacology* **36:** 27–38 (1992).
2. T.S.C. Li. Asian and American ginseng. A review. *Hort Technol.* **5:** 27–34 (1995).
3. S. Shibata, O. Tanaka , J. Shosi, and H. Saito. "Chemistry and pharmacology of *Panax*". In *Journal of Economics and Medicinal Plant Research.* Academic Press, London. U.K., pp. 217–84 (1985).
4. T. Furuya, T. Yoshikawa, T. Ishii, and K. Kajii. Regulation of saponin production in callus cuture of *Panax ginseng*. *Planta Med.* **47:** 200–204 (1983).
5. Y.K. Zhang and J.J. Zhong. Hyperproduction of ginseng saponin and polysaccharide by high density cultivation of *Panax notoginseng* cells. *Enzyme Microb. Technol.* **21:** 59–63 (1997).
6. A. Mathur, A.K. Mathur, M. Pal, and G.C. Uniyal. Comparison of qualitative and quantitative in vitro ginsenoside production in callus cultures of three Panax species. *Planta Med.* **65:** 484–86 (1999).
7. M. Bonfill, R.M. Cusido, J. Palazon, M.T. Pinol, and C. Morales. Influence of auxins on organogenesis and ginsenoside production in *Panax ginseng* calluses. *Plant Cell Tissue Organ Culture* **68:** 73–78 (2002).
8. Y.N. Zhuralev, V.P. Bulgakov, L.A. Moroz, N.I. Uvarova, V.V. Makhankov, G.V. Malinovskaya, A.A. Artyuko, and G.B. Elyakov. Accumulation of panaxosides in a culture of cells of *Panax ginseng* CA Meyer transformed with the aid of *Agrobacterium rhizo - genes. Dokl Bot Sci Akad SSR* **311:** 22–23 (1990).
9. C. Kevers, Ph. Jacques, Ph. Thonart, and Th. Gaspar. In vitro root cultures of *Panax ginseng* and *P.quinquefolium. Plant Growth Regul.* **27:** 173–78 (1999).
10. K.W. Wu, W.Y. Gao, S.H. Son, and K.Y. Pack. Improvement of ginsenoside production by jasmonic acid ad some other elicitors in hairy root culture of ginseng (*Panax ginseng* CA Meyer). *In Vitro Cell. Dev. Biol. Plant* **36:** 424–28 (2000).
11. I. Asaka, I. Li, M. Hirotani, Y. Asada, T. Yoshikawa, and T. Furuya. Mass production of ginseng (*Panax ginseng*) embryoids on media containing high concentrations of sugar. *Planta Med.* **60:** 146–48 (1994).
12. Y. Shoyama, X.X. Zhu, R. Nakai, S. Shirashi, and H. Kohda. Micropropagation of *Panax notoginseng* by somatic embryogenesis and RAPD analysis of regenerated plantlets. *Plant Cell Reports* **16:** 450–53 (1997).
13. C. Kevers, N. Le Gal, M. Monteiro, J. Dommes, and Th. Gaspar. Somatic embryogenesis of *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathways. *Plant Growth Regul.* **31:** 209–14 (2000).
14. C. Kevers, Th. Gaspar, and J. Dommes. The beneficial role of different auxins and polyamines at successive stages of somatic embryo formation and development of *Panax ginseng in vitro. Plant Cell Tissue Organ Culture* **70:** 181–88 (2002).
15. M. Monteiro, C. Kevers, J. Dommes, and Th. Gaspar. A specific role for spermidine in the initiation phase of somatic embryogenesis in *Panax ginseng* CA Meyer. *Plant Cell Tissue Organ Culture* **68:** 225–32 (2002).
16. *Deutsches Arzneibuch,* 10th ed. (DAB 10). Deutscher Apotheker Verlag, Stuttgart, Germany, 1994.

17. H.R. Schulten and F. Soldati. Identification of ginsenosides from *Panax ginseng* in fractions obtained by HPLC by field desorption mass spectrometry, multiple internal reflection infrared spectroscopy and thin-layer chromatography. *J. Chromatogr.* **212:** 37–39 (1981).
18. T. Murashige and F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15:** 473–97 (1962).
19. C. Kevers, G. Bare, Th. Gaspar, Ph. Thonart, and J. Dommes. "Optimisation of *Panax ginseng* liquid cell cultures for biomass accumulation and ginsenoside production". In *Liquid Culture Systems for in vitro Plant Propagation*. A.K. Hvoslef-Eide and W. Preil, Eds. Kluwer, Dordrecht, the Netherlands, 2004, in press.
20. P. Pietta and P. Mauri. Improved high-performance liquid chromatographic method for the analysis of ginsenosides in *Panax ginseng* extracts and products. *J. Chromatogr.* **356:** 212–19 (1986).
21. H. Kanazawa, Y. Nagata, Y. Matsushima, M. Tomoda, and N. Takai. Preparative high-performance liquid chromatography on chemically modified porous glass. Isolation of saponins from ginseng. *Chem. Pharm. Bull.* **38:** 1630–32 (1990).
22. W.C. Chuang and S.J. Shen. Determination of ginsenosides in ginseng crude extracts by high-performance liquid chromatography. *J. Chromatogr. A* **685:** 243–51 (1994).
23. X. Wang, J.T.A. Proctor, Y. Kakuda, R.A. Krishna, and P.K. Saxena. Ginsenosides in American ginseng : comparison of *in vitro* derived and field-gown plant tissues. *J Herbs, Spices and Medic Plants*, **6:** 1–10 (1999).
24. I. Asaka, I. Ichio, M. Hirotani, Y. Asada, and T. Furuya. Ginsenoside contents of plantlets regenerated from *Panax ginseng* embryoids. *Phytochem.* **36:** 61–63 (1994).
25. K.W. Yu, W.Y. Gao, S.H. Son, and K.Y. Pack. Improvement of ginsenoside production by jasmonic acid and some other elicitors in hairy root culture of ginseng (*Panax ginseng* C.A. Meyer). *In Vitro Cell Dev Biol-Plant* **36:** 424–28 (2000).
26. J.J. Zhong, X.D. Meng, Y.H. Zhang, and S.S. Liu. Effective release of ginseng saponin from suspension cells of *Panax notoginseng*. *Biotech. Techniques* **11:** 241–43 (1997).

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