Natural Product Sciences
10(4) : 152-167 (2004)

Review

Thin Layer Chromatography: Bioactive Metabolites of Components of Traditional Chinese Medicines by Intestinal Bacteria

Dong-Hyun Kim*

College of Pharmacy and East-West Pharmaceutical Research Institute, Kyung Hee University, Seoul 130-701, Korea

Abstract – Traditional Chinese Medicines (TCM) have attracted great interest in recent researchers as alternative medicines for incurable diseases. This review focuses on qualitative and quantitative analytical approaches for bioactive metabolites of components flavonoids and saponins of traditional Chinese medicines by TLC system, although various methods have been introduced. Emphasis will be put on the processes of metabolite extraction from intestinal bacterial cultures or urines, separation (mobile phase) and detection. The identified metabolites by selection of extraction solvent and detection methods are also discussed. In addition, metabolite determinations of flavonoids (baicalin, apiin, rutin, quercetin, quercitrin, kaempferol, diosmin, hesperidin, poncirin, naringin, puerarin, daidzin, daidzein, tectoridin) and saponins (ginsenosides, kalopanaxsaponins, glycyrrhizin, chiisanoside, saikosaponins, soyasaponins) in culture fluid, in urine and in some herbal formula extracts are summarized. These bioactive metabolites of these components by intestinal microflora should be connected to pharmacological actions.

Keywords – traditional Chinese medicines, flavonoid, saponin, metabolite, determination, TLC

Introduction

Traditional Chinese (or Oriental) medicine (TCM) arose from mythical medicine to a system of herbal medicine in China, Korea, Japan and Asia countries. It has been the unique tradition of TCM to summarize in writing the knowledge of herbs that people has gained over the past period of time. Since Shen Nong collected scattered records of herbal knowledge (Shen Nong Ben Cao Jing in Chinese), a lot of TCM literatures have been developed by continuous addition of new herbal medicines, together with a reevaluation and addition of new uses for the previously evaluated herbs (Zhu, 1988; Liu, 1987). The tradition continues into the modern era with the publication. Although herbal medicines have been recompiled many times, the basic contents remained the same. This is due to the fact that the evaluation of herbal medicines could only be recorded by famous and well-experienced authors. In other words, the effects of these herbal medicines have been tested and screened through the clinical experiences for many generations. By the process of trials, the worthless or less efficient ones were gradually eliminated. At present there are about 400 – 600 kinds of herbal medicines in daily use and about 150 of them are on the top list for scientific research.

*Author for correspondence
Fax: +82-2-961-0374; E-mail: dhkim@khu.ac.kr

Bioactive components of herbal medicines and intestinal bacteria

These herbs in TCM are formulated according to patient constitution and diseases (Lee, 1996; Liu *et al.*, 2002). Thus, there is no arbitrary mixture of herbs that might be useful in case of illness, but a formula devised according to strict rules. The technique of combining is the selection, for clinical purposes, of two or more herbal medicines with the idea of increasing or promoting therapeutic effectiveness, minimizing toxicity or side effects, accommodating complex clinical situations, and altering the major functions of herbal medicines. A typical prescription is composed of the principal medicine, the associate medicine, the adjuvant medicine and the messenger medicine. These herbal medicines are often described as emperor, minister, assistant and envoy in TCM literatures, respectively.

Recently, the effects of these herbs and their formulas have been tested and screened through *in vitro* biological activity assays rather than clinical experiences. Most traditional medicines are orally administered. However, a lot of components of these medicines such as glycosides saponins and flavonoid glycosides cannot be absorbed

from the intestine. Therefore, these components inevitably come into contact with intestinal microflora in the alimentary tract and can be metabolized by intestinal microflora. Then the metabolites are easily absorbed from the gastrointestinal tract, because most of the metabolites are nonpolar compared to the parental components. These absorbed metabolites express pharmacological actions (Kobashi and Akao, 1997; Wakabayashi *et al.*, 1998; Kim, 2002). Nevertheless, many researchers did not consider the metabolism of herbal medicine components by intestinal microflora for evaluation of their pharmacological actions. For example, glycyrrhizin (GL), which is a main component of licorice extract (*Glycyrrhiza glabra*), is ingested orally as a sweetener as well as being a component in Oriental medicine. GL shows various pharmacological actions including hepatoprotective, steroid-like, antiviral and anti-inflammatory activities (Kumagai *et al.*, 1957; Finney *et al.*, 1958; Tangri *et al.*, 1965; Conn *et al.*, 1968; Pompeo *et al.*, 1979; Abe *et al.*, 1982). By the oral administration of GL to human, 18β-glycyrrhetinic acid (GA) was detected in the sera, but GL was not (Nakano *et al.*, 1980). Hattori *et al.* (1983) and Kim *et al.* (1996, 1999 and 2000) reported that GL is transformed to GA by human intestinal bacteria. Kobashi and Akao (1997) reported that GL did not be metabolized and absorbed in blood in germ-free rats. The hepatoprotective activity of GL at a daily dose of 100 mg/kg for 3 days was observed in gnotobiotic hepatotoxified rats induced by carbon tetrachloride, however no activity was shown in germ-free group (Fujita *et al.*, 1978). Shim *et al.* (2000) also reported that oral administration of GL and intraperitoneal administration of GA showed the protection of hepatotoxicity of rats, but intraperitoneal administration of GL did not show the activity. These studies suggest that if GL is orally administered to human, it is converted to GA by human intestinal microflora before GL was absorbed into the body and GA exhibited the pharmacological actions. Therefore, to understand the pharmacological actions of herbal medicines, we must isolate and determine the metabolites of their main components and investigate their pharmacological actions.

Assay of bioactive components from herbal medicines

To understand the pharmacological actions of herbal medicines, many researchers extracted herbal medicines with water or organic solvents, and orally or intraperitoneally administered their whole extracts in animals. However, in connection with clinical experiences, what evaluate these studies is difficult. A lot of intact components of these extracts cannot be absorbed from intestine, because the components are hydrophilic. From these herbal medicines, identifying and isolating bioactive components are not easy by *in vitro* studies, because, after their components are metabolized to active compounds by intestinal microflora, most metabolites *in vivo* express pharmacological actions. Therefore, to evaluate pharmacological actions of the herbal medicines in human, we need to identify, isolate and determine the metabolites absorbable from intestine into blood by human intestinal microflora, and must investigate the relationship between metabolism of herbal medicine components and their biological activities.

Merit of thin layer chromatography (TLC)

To identify and isolate the metabolites of herbal medicine components, many researchers use many techniques, such as TLC-densitometry, gas chromatography (GC), high performance liquid chromatography (HPLC), spectrophotometry, liquid chromatography (LC)-mass, LC-NMR, capillary electrophoresis, *etc.* First three methods were generally used for qualitative and quantitative analysis of flavonoids and saponins of herbal medicines. However, GC has been used within the limit of volatile compounds such as equol, because most flavonoids and saponins are not volatile compounds. HPLC is an accurate and good-recovery tool of identifying and assaying the components of reaction mixtures. However, it is complex and time-consuming for metabolite analysis of herbal medicine components by intestinal microflora.

TLC is a simple and time-saving tool of identifying the components of a mixture, monitering the course of a metabolic reaction, estimating the metabolic pattern, or isolating trace amount of bioactive components (Maugh, 1982; Poole, 1999, 2000, and 2003). Nevertheless, TLC and HPLC must be viewed as complementary techniques that can be distinguished by their different attributes. Particularly, in many study of qualitative and quantitative metabolic analysis of herbal medicine components, TLC can be first chosen rather than HPLC. Based on these merits, the present review focuses TLC application in qualitative and quantitative analysis of bioactive metabolites of herbal medicine components.

Most TLC analyses are performed on plates coated with silica gel or modified silica: most of the rest are performed on plates coated with alumina, cellulose, or modified celluloses. On a conventional TLC plate, about 20 samples can be chromatographed in about the same time required for one HPLC analysis, and at less cost. Standards are run at the same time on the same plate, so there is greater assurance of the identity of separated

components. The pretreatment (clean-up) of the sample for loading on TLC is simple. Most metabolites of herbal medicine components may foul a much more expensive HPLC column. Therefore, the sample must be cleaned up before it is injected onto an HPLC column. TLC is especially useful compared to HPLC. The technique is widely used.

For many years, the silica used for TLC plates had a wide range of particle diameters, and results were occasionally unpredictable. Recently plate manufacturers began using the ultrafine silica particles, between 2 and 5 micrometers in diameter, which were developed for high performance thin layer chromatography (HPTLC) (Maugh, 1982; Jost and Hauck, 1987). These new plates were developed for HPTLC. The plate with the small particle diameters separates various compounds in much smaller distances, with high resolution, and in shorter times. Also HPTLC plates give more reproducible separations and the detection limits are much lower.

Metabolic fecal microflora preparation – To identify, isolate and determine the metabolites of herbal medicines by intestinal microflora, at first fresh intestinal microflora should be prepared. Mistuoka *et al.* reported that number and species of intestinal microflora are almost same to those of fresh fecal microflora (Mitsuoka, 1980). Therefore, Hattori *et al.* (1985), Kim *et al.* (2002), and Kobashi and Akao (1997) used filtrated fecal suspension for metabolic analysis of herbal medicines. Nevertheless, they did not compare the preparation methods of fecal suspension. Lee *et al.* prepared the fecal suspension by various methods, and measured the metabolic activities of herbal medicine components to bioactive compounds (Lee *et al.*, 2002). The difference was not significant between the preparation methods. Therefore, we frequently used the fecal suspension prepared by centrifugation, because the procedure is convenient and excluded a lot of interfering fecal components.

Extraction of metabolites of herbal medicine components

Extraction is the first important step for the recovery and purification of herbal medicine components and their metabolites. The traditional techniques of solvent extraction are mostly based on the correct choice of solvents and the use of heat and/or agitation to increase the solubility of compounds and the rate of mass transfer. Recently high intensity or power ultrasound are also applied in the extraction of various phytochemicals, such as flavonoids, saponins, and alkaloids (Li and Zhang, 1996).

In order to extract the metabolites from urines or culture fluids of intestinal microflora, fecal microflora and the properties of herbal medicinal components must be understood. The biological fluids are complex mixtures, composed of protein, lipid, amino acid and electrolytes, small molecules and intact bacteria. Beyond the deleterious effect that these compounds may cause on sample spotting and separation, their presences will frequently interfere with the separation of these metabolites. Consequently, selection of the sample preparation is always required before sample spotting onto the TLC system. For the quantitation of metabolites in TLC, sample pretreatment, including bacteria precipitation, liquid-liquid phase extraction (if necessary, solid-phase extraction on cartridges), or dilution of sample in an appropriate solvent of buffer have been used. The choice of pretreatment strategies and sample clean-up procedures will affect the efficiency and the selectivity of TLC technique.

Liquid-liquid extraction (Li and Zhang, 1996; Tsai, 2001) – In liquid-liquid phase extraction, an immiscible organic phase is added to the reaction mixture. These mixtures are then shaken together, resulting in the more hydrophobic sample components being extracted into the organic phase such as ethyl ether, dichloromethane, ethyl acetate and butanol. The bacteria and inorganic salts present at high concentrations in most reaction mixtures are absent from the extract. This strategy is very useful from the viewpoint of metabolite polarity. The application of liquid-liquid phase extraction in the simultaneous determination of intact and metabolic components of herbal medicines are very valuable: the extraction efficiencies were more than 90%, if organic solvents were appropriately selected, although the extraction rates of some metabolites in urine are not sufficient.

Liquid-solid phase extraction (Ahmed *et al.*, 1998; Kurita and Kaneda, 1999) – Differences in affinity of various compounds to active sites located on the surface of sorbent materials resulting in separation of mixtures of different compounds are the basic theory of solid-phase extraction. The first step of the procedure is adsorbing metabolites into either an ion-exchange resin, if they exhibited charge, or bonded reversed-phase packing materials. With respect to the type of the bonded phases, octadecyl-bonded silica is the most popular phase. Others, such as C2, C4, C8, phenyl, etc were introduced for various extractions. In the quantitative analysis of metabolites, if the strength of interaction in packing materials between metabolites of herbal medicines and waste components were applied well, the extraction efficiencies were about 100%. However, application of the liquid-solid extraction in metabolic studies of herbal

medicines is too difficult, because the metabolites of most components of herbal medicine are unknown.

Separation and detection – Liquids used in liquid-liquid phase extraction and solid-phase extraction can be evaporated away, and the residues were then redissolved in a small volume of liquid. Samples for analysis are typically applied in a row near the bottoms in diameter, with solvent volumes of 0.1 to 5 μl. The plate is then placed in a closed chamber with the bottom resting in a shallow pool of eluent. The eluent or mobile phase in TLC works its way up the plate by capillary or wicking action in a manner similar to that occurring in HPLC. The two techniques are so similar. TLC can be used to identify the optimum conditions for HPLC.

Sample size is so important for quantitation in TLC. The sample of uniform size can be spotted onto plates by hand, but it is a tedious business that requires skill and patience. Therefore, a whole new line of automatic spotting equipment has been developed by many researchers. The automated spotters, in general, do a very good job of applying a precise amount of sample exactly where it is wanted. Their chief disadvantages in the metabolite analysis of herbal medicne components are that they are often difficult to clean and require preconcentration of the sample.

The separation of herbal medicine components and their metabolites using TLC has often required appropriate developing solvents. Many researchers have been used diverse developing solvents for flavonoids, saponins and their metabolites (Oleszek, 2002). Some solvents are introduced in the below. Once the chromatogram is well developed, it is necessary to visualize the normally colorless spots. At first, the chromatogram was visualized under UV light. And then the time-honored way of doing this is to place the plate in a bottle containing iodine crystals, whose vapor will turn many compounds reddish-brown. Another common technique is to impregnate the plate with specific reagents, such as 5 – 10% sulfuric acid in ethanol, p-anisaldehyde-sulfuric acid in alcohol and inorganic phosphors.

The final step in the TLC process is generally qualitative or quantitative determination of the amount of material in each spot. Most commonly, a densitometer is used to moniter the difference in ultraviolet or visible absorption between the background and the spots or the difference in phosphorescence, to determine the relative sizes of the spots (Baccou *et al.*, 1977; Poole and Poole, 1989; Prosek *et al.*, 2002). In certain case, retrieval of the sample may be desired. This can be accomplished by scraping off the layer of adsorbent containing the spot or by extracting the spot *in situ* (colorimetry). However, the disadvantage of this method is poor recovery. Recently TLC-videometry is occasionally used (Prosek *et al.*, 2002).

Flavonoids

The flavonoids which occur both in the free state and as glycosides are the largest group of naturally occurring phenols (Evans, 2002). More than 2000 of these compounds are now known, with nearly 500 occurring in the free state. Most are O-gycosides, but a considerable number of flavonoid C-glycosides are known. Dimeric compounds are also known. They are classified into flavones, isoflavones, flavonols, flavanones, biflavonols, anthocyanins, *etc.*

The flavonoids are often yellow (Latin *flavus*, yellow). They are widely distributed in nature but are more common in the higher plants and in young tissues, where they occur in the cell sap. They are abundant in the Polygonaceae, Rutaceae, Leguminosae, Umbelliferae and Compositae.

Metabolic and pharmacological aspects

Although the original high hopes for the therapeutic useful of flavonoids were not immediately realized, recent researches have demonstrated their involvement in the pharmacological action of herbal medicines (Evans, 2002; Middleton *et al.*, 2000). The pharmacological action of the flavonoids is known for antiinflammatory, antiallergic effects, antithrombotic, vasoprotective properties, inhibition of tumor promotion and gastric mucosa protective. These effects have been attributed to the influence of flavonoids on arachidonic acid metabolism. Some flavonoid-containing plants are diuretic or antispasmodic. Some flavonoids have antitumor, antibacterial or antifungal properties. Most isoflovones are known for non-steroidal phytoestrogens.

These flavonoids and their glycosides are polyphenolic compounds produced by most herbal medicines as well as vegetables. These compounds are ingested daily more than 1 g by human, but are resistant to boiling and fermentation. After ingestion of flavonoid glycosides, most of them are not easily absorbed in intestinal gut. Therefore, the components of these medicines inevitably come into contact with intestinal microflora in the alimentary tract and can be metabolized by intestinal bacteria. Evidence for the involvement of the intestinal microflora in the metabolism of flavonoid compounds *in vivo* has been presented in animals and humans by Griffiths group (1964, 1972a, 1972b), Rechner group

(2002) and Manach group (1977, 1995). Particularly, it was reported that the ring fissuring as well as glycosidation from orally administered flavonoids were significantly decreased by the coadministration of oral antibiotics. The metabolites from the urine of rats orally treated with flavonols, such as quercetin and kaempferol, and their glycosides were aglycones as well as phenolic acids. When flavonoid glycosides, such as rutin, naringin, poncirin and hesperidin, were anaerobically incubated with human intestinal microflora, they were metabolized to their aglycones and then to phenolic acids (Kim *et al.*, 1994, 1998; Bokkenheuser *et al.*, 1987). For example, rutin was transformed to 3,4-dihyroxyphenylacetic acid, 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid *via* quercetin by human intestinal microflora. The rutin glycosidating bacterium was *Bacteroides* JY-6, which potently produced α-rhamnosidase and β-glucosidase. Quercetin C-ring was fissured and metabolized to phenolic acids by *Pediococcus* Q-5 and *Bacteroides* JY-6.

Related to the relationship between biological activities and metabolism of flavonoids, Knekt *et al.* (2002) reported that quercetin intake decreased mortality for ishcemic heart disease and lung cancer incidence, kaempferol intake reduced the indicence of cerebrovascular disease, and naringenin and hesperetin reduced the athma incidence. The quercetin is an aglycone produced from quercitrin, rutin, *etc* by intestinal bacteria. Hesperetin, naringenin and ponciretin are an aglycone produced from flavanone glycosides hesperidin, naringin and poncirin by intestinal bacteria, respectively. Therefore, orally administered quercetin and quercetin glycosides may exhibit similar pharmacological actions *in vivo*. Youn *et al.* (1992) reported that antiinflammatory activity of oral administration of poncirin isolated from the fruit of *Poncirus trifoliata* was originated from ponciretin metabolized by intestinal microflora. Kim *et al.* (1998) reported that metabolites 3,4-dihydroxyphenylacetic acid and 4-hydroxylphenylacetic acid of quercetin and ponciretin showed the potent antiplatelet aggregation activities. The phenolic acid metabolites of flavonoids also exhibited cytotoxic actions against tumor cell lines. On the preventive effect of flavonoid glycosides for chronic illness (stoke, tumor and inflammation), the metabolism of flavonoid glycosides to their aglycones and phenolic acids by intestinal bacteria may be important.

Analytical (qualitative and quantitative) methods

Flavones – Baicalin, apigenin, tricetin diosmin and luteolin are the representative flavones. Apigenin, apiin and acacetin are common components of flos of *Daphnis genkwa*, flos of *Buddleia officinalis*, flos of *Chrysanthemium indicum*, and seed of *Perilla frutescens* (Hsu *et al.*, 1982).

Griffiths and Smith studied the metabolism of apigenin, apiin, acacetin and 4,7-dihydroxyflavone *in vivo* after oral administration in rat, and *in vitro* in the culture of microorganisms derived from the intestine of the rats (Griffith and Smith, 1972). They analyzed the metabolites of these compounds in feces and urine by using TLC systems. The urine samples were acidified and extracted with ethyl ether. The fecal samples were freeze-dried, then extracted with hot methanol, and filtered. The extracted samples were evaporated to dryness and dissolved in methanol. Samples of the methanolic solutions of extracts of both urine and feces were then submitted to TLC. The efficiency of the standard extraction methods for flavonoid metabolites was determined by adding known amounts of each to samples of control urine and feces. When the compounds were then extracted and quantitatively measured, the recovery of phenolic acids and kaempferol from urine was greater than 60%, but the recovery of apigenin from urine was about 20%. By contrast, the recovery of metabolites from feces was always greater than 90%. They used benzene-pyridine-formic acid (35:9:5), chloroform-acetic acid-water (2:3:1), chloroform-acetic acid-water (4:1:1), benzene-dioxane-acetic acid (90:25:4) as mobile phases, and determined metabolites of flavanoids under UV light or after staining with diazotized p-nitroaniline or diazotized sulfuric acid, *etc*. In the case that these methods could be not available, they scraped the metabolites for the plates and determined the metabolites by the Folin-Ciocalteu method. By using this method, they identified and quantitatively analyzed p-hydroxyphenylpropionic acid, p-hydroxycinnamic acid, p-hydroxybenzoic acid and apigenin in urine of rats orally administered apigenin. In cultured fluid of apigenin with intestinal microflora, p-hydroxyphenylpropionic acid was identified and determined. When apiin was orally administered to rats, they also analyzed p-hydroxyphenylpropionic acid, p-hydroxycinnamic acid, p-hydroxybenzoic acid and apigenin in urine, and p-hydroxyphenylpropionic acid, apigenin and apiin in feces. In cultured fluid of apiin with intestinal microflora, p-hydroxyphenylpropionic acid was identified and determined. They also identified and determined p-hydroxyphenylpropionic acid, apigenin and acacetin in the urine of rats orally administered acacetin. However, the limit of determination of these metabolites is not reported.

Griffith and Smith also investigated the metabolism of

tricetin in rats by the above method (Griffith and Smith, 1973). They identified 3,5-dihydroxyphenylpropionic acid in urine. When tricetin was incubated with rat intestinal microflora, 3,5-dihydroxyphenylpropionic acid and 3-hydroxyphenylpropionic acid were identified.

Baicalin is a main component of the rhizome of *Scutellaria baicalensis*, which have been used as an antioxidant, anti-inflammatory and antiischemic agent (Chen *et al.*, 2001; Li *et al.*, 2000). Akao *et al.* (2000) orally administered baicalin or water extract of Scutellariae Radix to rats, and analyzed baicalin and baicalein in blood by TLC and HPLC systems. They suggest that baicalin may be metabolized to baicalein by intestinal microflora. Thereafter they identified baicalein in culture fluids of intestinal microflora with baicalin. However, they did not determine phenolic metabolites of flavonoids due to a lot of endogenous and exogenous compounds. Kim *et al.* also reported that in culture fluid of human intestinal microflora with baicalin, baicalin was metabolized to baicalein, which then is progressively metabolized to 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (procatechuic acid), pyrogallol and phenylacetic acid in intestine or in intestinal bacteria culture. They used chloroform-acetic acid-water (lower layer) (2:1:1) and chloroform-methanol (4:1) as mobile phases (Kim *et al.*, 1996, 1998).

When Kuroki and Poulton assayed apigenin para-O-methylation activity of isolated bacteria, they used benzene-dioxane-acetic acid (90:25:4) and toluene-ethyl acetateacetic acid (9:2:0.5) as mobile phases for the separation of apigenin and acacetin (silica IB-F thin layer sheets, J.T. Baker Chem. Co. N.J.) (Kuroki and Poulton, 1981). In simple reaction mixtures like this experiment, the metabolites may be easily and quickly determined by TLC system than by HPLC system.

Zheng *et al.* (1996) analyzed the content of baicalin in herbal medicinal formula to control quality of the herbal formula preparation. They extracted Tanre Kesou Granules, which contained Scutellariae, Fritillariae Radix, Thunbergii Bulbus, Citri reticulatae Pericarpium and Aurantii immaturus Fructus, with ethylacetate and assayed baicalin by TLC-densitometry. Its recovery was >98%. However, the limit of determination of these metabolites is not reported.

Flavanols – Rutin, quercetin, quercitrin kampferol and myricetin are the representative flavanols and are contained in flos of *Sophora japonica*, cortex of *Myrica rubra*, herba of *Houttuynia cordata*, herba of *Orostchys japonicus* (Hsu *et al.*, 1982). These flavanols exhibited potent cytotoxic, angiogenic and antioxidant actions. Tamura *et al.* (1980) reported reported that quercetin showed mutagenic activity by Ames test. Thereafter, many researchers reported that these compounds did not only exhibit the mutagenic action, however, showed the anticarcinogenic actions (Stoewsand *et al.*, 1984; MacGregor, 1979). Related to these findings, Kim *et al.* (1996) reported that, when the metabolites were analyzed in urine of rats orally administered rutin and in culture fluid of quercetin or rutin with intestinal bacteria, quercetin, phloroglucinol, 3,4-dihydroxybenzoic acid were detected as metabolites by TLC system: mobile phases were chloroform-acetic acid-water (2:1:1, lower layer), benzene-acetone-chloroform-methanol (8:2:3:2), and chloroform-methanol (4:1). Kim *et al.* (1999) also analyzed the metabolite quercetin of quercitrin, which is a representative component of *Houttuynia cordata*, by TLC: developing solvent, chloroform-methanol-water (65:35:10, lower layer) and chloroform-methanol (4:1); detection, TLC scanner under UV light. They can periodically analyze the amount of quercetin a main metabolite by human intestinal microflora. The limit of determination of these metabolites is not reported.

Griffith and Smith (1972) reported that in the urine of rats orally administered kaempferol, p-hydroxyphenylacetic acid, kaempferol were detected by TLC system. When robinin was orally administered to rats, p-hydroxyphenylacetic acid and kaempferol were detected in urine, and p-hydroxyphenylacetic acid and kaempferol were in faeces. However, the limit and recovery of determination of these metabolites are not reported. They also identified and determined *in vivo* the metabolites of myricetin and myricitrin by using the above method for extraction and separation of metabolites in TLC (mobile phases, developing solvents for metabolite determination of the above apigenin). 3,5-Dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid were identified in the urine of rats orally administered myricetin. Myricetin, 3,5-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid were identified in the urine of rats orally administered myricitrin. Myricetin, 3,5-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and 3,4,5-trihydroxyphenylacetic acid were identified in culture fluid of myricetin with fecal microflora.

In early study Booth *et al.* (1956) reported that, in the urine of rats orally administed rutin and quercetin, quercetin, 3-hydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid were identified as metabolites by TLC system.

To identify the metabolites of rutin and quercitrin by fecal and saliva enzymes and to measure the mutagenicity

of their metabolites, MacDonald *et al.* (1983) analyzed their metabolites by TLC system. The reaction mixtures in the plate were chromatographed in toluene-ethylacetate-methanol-formic acid (32:14:12:5), and analyzed under UV light. The limit and recovery of determination of these metabolites is not reported.

Krishmamuruy *et al.* (1970) incubated rutin and quercetin with *Butyrivibrio spp.* C3, extracted with ethyl acetate and investigated the metabolites. They identified 3,4-dihydroxyphenylacetic acid by using chloroform-acetic acid-water (2:1:1) as a mobile phase and silica gel F254 as a stationary phase.

For the extraction and identification of flavonoid (quercitrin and kaempferol) metabolites by flavonoid C-ring cleavage intestinal bacteria (Clostridia), Bokkenheuser *et al.* (1987) and Winter *et al.* (1989) used TLC and HPLC analytic methods. The culture fluids of flavonoids with intestinal bacteria were acidified to pH 2 and extracted with ethylacetate. The organic phase was collected, dried over sodium sulfate, and evaporated under nitrogen at 40 – 45°C. We also frequently use this method. In this system, organic phase must evaporate under nitrogen at less than 45°C. The extracted residue was redissolved in MeOH and spotted onto silica gel plate (IB2F Flex; J.T. Baker Chemical Co.). The metabolites were developed by using benzene-acetic acid-water (83:48:2) as a mobile phase. The developed plates were detected and analyzed under UV lamp and by idodine vapor staining. They identified 3,4-dihydroxyphenylacetic acid as a metabolite of quercitrin, and 4-hydroxy phenylacetic acid as a metabolite of kaempferol.

Flavanones – Hesperidin, neohesperidin, naringin and poncirin are the representative flavanones and are main components of *Citrus spp.* and *Poncirus spp.*, which are used as digestive, anti-inflammatory and antiarteriosclerosic agents (Evans, 2002; Hsu *et al.*, 1982).

Griffiths and Smith (1972) orally administered naringin to rats and investigated its metabolites in the urine. They identified p-hydroxyphenylpropionic acid, p-hydroxy-cinnamic acid, p-hydroxybenzoic acid and naringenin by TLC, and in feces, p-hydroxyphenylpropionic acid was identified. In culture solution of naringin with fecal bacteria, p-hydroxyphenylpropionic acid was detected as a metabolite.

Cheng *et al.* (1971) fermented naringin with *Butyrivibrio spp.* to obtain sufficient quantities of products for chemical confirmation studies and analyzed the metabolites by TLC system. To analyze the metabolites, the cultured fluids were adjusted to pH 2 with HCl and centrifuged at 13200×g for 20 min. The supernatant was decanted and then lyophilized and the dry residue was extracted by refluxing for 24 h with ether. The ether extract was treated with petroleum ether to remove fatty acids, concentrated and then analyzed metabolites. They identified p-hydroxyphenylpropionic acid, naringenin and phloroglucinol by TLC system [mobile phase, butanol-acetic acid-water (6:1:2) and acetic acid-water (15:85)].

Winter *et al.* (1989) analyzed the metabolites of naringin as well as quercitrin by using the above method. They used benzene-acetic acid-water (83:48:2). The spots of flavonoids and metabolites were detected under UV lamp and by idodine vapors.They identified phenylacetic acid, phloroglucinol as metaboltes.

Kim *et al.* (1998) reported that in the culture solution of naringin, hesperidin or poncirin with intestinal bacteria, naringenin, 4-hydroxybenzoic acid, phloroglucinol and p-coumaric acid for naringin, hesperetin, resorcinol, phloroglucinol and caffeic acid for hesperidin, and ponciretin, 4-hydroxybenzoic acid, 2,4-hydroxyacetophenone and phloroglucinol for poncirin were detected. However, amounts of these metabolites were not quantitatively determined. Recently to understand the metabolic pathways of hesperidin, poncirin and naringin by human intestinal bacteria, Kim *et al.* (1994, 1998) determined periodically the metabolite naringenin and ponciretin in reaction mixtures by using TLC system: mobile phase, chloroform-methanol (4:1); coloring agent, 5% sulfuric acid in alcohol. However, the limit and recovery are not reported.

Isoflavones – Puerarin, daidzein, tectorigenin, irisolidone and genistein, which are the representative isoflavone, were contained in flos of *Pueraria thunbergiana*, and rhizome of *Pueraria thunbergiana*, rhizome of *Glycyrrhiza glabra* and *Glycine max* (Family Leguminosae) (Hsu *et al.*, 1982). These herbs have been frequently used as osterogenic and cancer-preventive agents in TCM (Nohara and Kinjo, 1984; Hayakawa *et al.*, 1984). When the contents of isoflavone in the rhizome of *Puararia thunbergiana* were determined by TLC, they used chloroform-methanol-water (65:35:10, lower layer) under UV light (λs 250 nm, λr 320 nm), and Kim *et al.* (1998, 1999) also determined for the metabolites of tectoridin, glycitin, puerarin, daidzin and kakkalide by human intestinal bacteria by TLC system using chloroform-methanol or chloroform-methanol-water (65:35:10, lower layer) as a mobile phase under UV light. Moreover, they identified daidzein, 4-hydroxybenzoic acid, 2,4-hydroxy-benzoic acid, resorcinol and 2,4-dihydroxyacetophenone as metabolites of puerarin and daidzin. Griffith and Smith (1972) also investigated the metabolite of genistein and

daidzein in urine of rats orally administered these isoflavones by using the above methods. They identified p-ethylphenol for genistein and equol as daidzein. Now the metabolite equol of isoflavones by intestinal bacteria have been frequently determined by GC (Morton *et al.*, 2002). However, the phenolic metabolites were not measured. Kim *et al.* (1998, 1999) did not also quantitatively determine these metabolites. Although Griffiths and Smith (1972) measured the content of the metabolites, the limit of determination of these metabolites was not investigated.

Saponins

Saponins have a high molecular weight and a high polarity (Evans, 2002; Hsu *et al.*, 1982). Often they occur as complex mixtures with the components differing only slightly from one another in the nature of the sugars present, or in the structure of the algycone (sapogenin). According to the structure of the aglycone or sapogenin, two kinds of saponins are recognized - the tetracyclic (common steroidal triterpenoids) and the pentacyclic triterpenoid types. Both triterpenoid saponins have a glycosidal linkage at C-3. A distinct subgroup of the steroidal saponins is that of the steroidal alkaloids which characterize members of the Solanaceae. They possess a heterocyclic nitrogen-containing ring.

The steroidal saponins are less widely distributed in nature than the pentacyclic triterpenoid type. These saponins are contained in many monocotyledonous families, particularly the Dioscoreacease, Agavacease, and Smilacacease. In the dicotyledons diosgenin occurred in Leguminosae and Araliaceae. Some species of *Strophanthus* and *Digitalis* contain both steroidal saponins and caridoglycoside. Rare steroidal alkaloids are discovered in *Solanum* (Solanceae).

Unlike the steroidal saponins, the pentacylic triterpenoid saponins are rare in monocotyledons. They are abundant in many dicotyledonous families, particularly the Caryophylaceae, Sapindaceae, Polygonaceae, Sapotaceae, Phytolaceae, Chenopodiaceae, Ranunculaceae, Berberidaceae, Papaveraceae, Linaceae, Araliaceae, Zygophyllaceae, Rutaceae, Myrataceae, Cucurbitaceae, Umberlliferae, Rubiaceae, Compositae, and Oleaceae.

These triterpenoid saponins are classified into dammarane, oleanane, ursane, lupane types, *etc*. Sugar or uronic acid units, or both, often are bound in the 3-position of triterpenoid sapogenins. The related triterpenoid acids are formed from these compounds by replacement of a methyl group by a carboxyl group in positions 4, 17 or 20, which is modified by glycosyl groups.

Metabolic and pharmacological aspects

The pharmacological action of the saponins is known for hemorrhagic, antiinflammatory, antiallergic and antitumor effects (Evans, 2002). Some saponins have antibacterial, antifungal or phytoestrogenic properties.

Most saponins are triterpenoid glycosides produced by most herbal medicines as well as vegetables. After ingestion of herbal medicines, most saponin glycosides in herbal medicines are not easily absorbed in intestinal gut. Therefore, these components inevitably come into contact with intestinal microflora in the alimentary tract and can be metabolized by intestinal bacteria. Evidence for the involvement of the intestinal microflora in the metabolism of saponins *in vivo* has been presented by Shimizu group (1985) and Kanaoka group (1994).

For example, saikosaponin a, c, and d were main components of rhizome of *Bupleurum falcatum*, which is used for treatment of liver diseases and inflammation. Related to the metabolism of saikospanin a, Shimizu *et al.* (1985) reported that saikosaponin a was transformed into 2 structural isomers by incubation with rat gastric juice, and that these saikosaponin diglycosides were changed into monoglycoside and algycones by anaerobic incubation with mouse intestinal microflora *in vitro*. Saikosaponin c and d are also similarly metabolized to that of saikosaponin a. In *in vivo* study, monoglycoside and aglycone of saikosaponin a were detected in blood of rats orally administered saikosaponin a. Compared to biological activities of saikosaponin a and its metabolites, metabolites showed more potent protein biosynthesis activity.

Ginsenosides are main components of ginseng (the root of *Panax ginseng* C.A. Meyer, Araliaceae), which is frequently used as a crude substance taken orally in Asian countries as a traditional medicine as well as a functional food. Ginsenosides have been reported to show various biological activities including anti-inflammatory activity and anti-tumor effects (inhibition of tumor-induced angiogenesis and prevention of tumor invasion and metastasis) (Wu *et al.*, 1992; Lee *et al.*, 1999; Choo *et al.*, 2003). However, the cytotoxic ginsenosides were not isolated from ginseng. When ginseng was orally administered to humans and rats, compound K (20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol) in the plasma was detected (Akao *et al.*, 1998; Kanaoka *et al.*, 1998a). To solve this clue, many researchers studied the metabolism of ginseng saponins by intestinal bacteria. When protopanaxadiol ginsenosides were incubated with human intestinal microflora, compound K was identified

as a main metabolite (Kanaoka *et al.*, 1998b; Hasegawa *et al.*, 1996; Bae *et al.*, 2000). The protopanaxadiol saponins were easily transformed to ginsenoside Rg3 under mild acid condition (Han *et al.*, 1982; Bae *et al.*, 2002). This ginsenoside Rg3 was transformed to ginsenoside Rh2 by human intestinal bacteria. This result suggests that protopanaxadiol saponins can be metabolized to compound K in the intestine by intestinal microflora and to ginsenoside Rh2 by gastric juice and intestinal bacteria. These metabolites exhibit many kinds of pharmacological actions.

Therefore, to express many kinds of pharmacological actions of ginseng in humans, it is believed that ginseng saponins may be metabolized by human intestinal microflora after being taken orally. The compound K transformed for protopanaxadiol ginsenosides shows *in vitro* and *in vivo* an antimetastatic or anticarcinogenic effect by blocking tumor invasion or preventing chromosomal aberration and tumorigenesis (Kime *et al.*, 2002; Lee *et al.*, 1999). Hasegawa *et al.* (2000) reported that the compound K is absorbed to blood and then metabolized to oleoyl compound K in liver by using TLC system. Among ginsenosides and their metabolites, compound K and 20(*S*)-protopanaxadiol showed potent cytotoxicity against tumor cell lines, and oleoyl compound K inhibited the metastasis of tumor cells. When the antiallergic activity of ginsenosides was evaluated, ginsenoside Rh2 and compound K showed the most potent inhibitory activity. Most saponin glycosides did not show pharmacological actions, but, if metabolized by intestinal microflora, showed the biological activity.

Analytical (qualitative and quantitative) methods

Dammarane derivatives – Ginseng has been used as a tonic and anticancer agent. Shibata *et al.* reported that its main components are ginsenosides (Shibata *et al.*, 1966). However, many researchers could not find antitumoral components by *in vitro* study. To solve this clue, many researchers studied the metabolism of ginseng saponins by intestinal bacteria. When protopanaxadiol ginsenosides were incubated with human intestinal microflora or isolated bacteria (*Provetella oris*, *Fusobacterium* K-60 and *Bifidobacterium* K-506), a main metabolite is found to be compound K by TLC system (chloroform-methanol-water (65:35:10, lower layer) as a mobile phase) (Hasegawa *et al.*, 1996; Bae *et al.*, 2000; Karikura *et al.*, 1990). The protopanaxadiol saponins were easily transformed to ginsenoside Rg3 by the treatment of mild acids (Han *et al.*, 1982). This ginsenoside Rg3 was transformed to ginsenoside Rh2 by human intestinal bacteria (Bae *et al.*, 2002). They determined metabolites by using chloroform-methanol-water as a mobile phase.

To assay the enzyme activity for purification of β-glucosidase of fresh ginseng, Zhang *et al.* (2001) also determined the biotransformants of ginsenoside Rg3 using chloroform-methanol-water (65:35:10, lower layer) as a mobile phase and TLC scanner as a detector. They identified ginsenoside Rh2. Using the same mobile phase, Liu *et al.* (2001) anaylized the metabolites of ginsenoside Rg1 by intestinal flora. They identified ginsenoside Rh1 and protopanaxatriol by TLC-electron spurt ion mass. Dong *et al.* (2003) screened ginsenoside Rb1-metabolizing microbes from *Rhizopus* spp. and *Curvularia* spp. and isolated four metabolites. The metabolites were qualitatively analyzed by TLC system [Silica gel 60GF254 (Qingdao Marine Chemical Factory, China); developing solvent, chloroform-methanol-water (5:5:1, lower layer); coloring agent, 10% sulfuric acid in ethanol].

To investigate contents of saponins in ginseng or to understand these metabolic pathways of ginseng saponins by human intestinal microflora, many researchers extracted saponins with butanol, methanol or water and then analyzed saponins and their metabolites by TLC analytic method. If the extracts are concentrated at higher temperature than 80°C, chemical transformants can be produced. Thus glycosyl moiety of 20-hydroxyl group of protopanaxadiol or protopanaxatriol could be hydrolyzed under acidic condition (Han *et al.*, 1982; Bae *et al.*, 2002). Therefore, when extracts are concentrated, you must keep them at less than 80°C. The concentrates are dissolved in methanol, spotted into TLC plate, and developed.

Many researchers used chloroform-methanol-water (65:35:10, lower layer) system as a developing solvent for the seperation of ginsenosides (Hasegawa *et al.*, 1996; Bae *et al.*, 2000; Bae *et al.*, 2002; Karikura *et al.*, 1990). This developing solvent was used for separation of ginsenosides, and their metabolites by intestinal microflora as well as liver. However, this developing solvent could not well-separate ginsenoside Rb2 and Rc, ginsenoside Rg1 and Rg3, and ginsenoside Ra1, Ra2, and Ra3. Therefore, to separate ginsenosde Rb2 and Rc, n-butanol-ethyl acetate-water (15:1:4) was used. To separate ginsenoside Rg1 and Rg3, Corthout *et al.* (1999) used chloroform-ethyl acetate-methanol-water (15:40:22:9) system as a mobile phase and could separate polar ginsenoside Ra, Rb1, Rb2, Rc and Rd by using this developing solvent. However, the metabolites compound K and ginsenoside Rh2 could not be separated well.

Ginsenoside spots on TLC could not be identified by UV or visible wavelength. Therefore, to identify these

ginsenosides, developed TLC plates should be generally dipped into 5% sulfuric acid in ethanol, heated at 105°C for 1 min. The colored plates were detected under 380 nm. The colored plates were sometimes dipped into liquid paraffin/hexane (1:2) and detected under UV 366 nm. These methods are quick and straightforward, and a complete validation was carried out. The recovery and detection limit of ginsenosides were greater than 99% and 7 – 10 ng/spot, respectively. Kanaoka *et al.* (1994) detected TLC spots by spraying with 1% CeSO4-10% sulfuric acid solution followed by heating 150°C for 3 – 4 min. However, the limit and recovery were not measured.

Yip *et al.* (1974) extracted ginseng with methanol, concentrated, then dissolved in water, partitioned first with petroleum ether and then with water-saturated butanol, and quantitatively determined ginsenoside composition of the extracts by TLC in four solvent systems (chloroform-methanol-water (65:35:10, lower layer), butanol-ethyl acetate-water (4:1:5, upper layer), chloroform-butanol-methanol-water (20:40:15:20, lower layer) and chloroform-methanol-ethyl acetate-water (2:2:2:1, lower layer). Detection was by sulfuric acid and heating. The second solvent resolved most of the ginsenosides except the distinctions between ginsenoside Rb1 and Rb2, and between ginsenoside Rg1 and Rg2. Although the first was not able to resolve ginsenoside Rb2 and Rc, this was compromised by the fourth, which showed preferentially a clear pattern of the panaxatriol group.

To analyze contents of saponins in ginseng roots and cultured ginseng cells, Wu *et al.* (2001) extracted these ginsengs with water or alcohol by ultrasound assistance, and the ginseng saponins extracted was applied to TLC plate (silica gel 60F254) with chloroform-methanol-water (15:12:2, lower layer) or butanol-ethyl acetate-water (4:1:5) as a mobile phase. The saponin spot was scraped off and mixed with 0.2 ml of acetic acid containing 5% vanillin and 0.8 ml of perchloric acid at 60°C for 15 min. The concentration of saponins was determined with a spectrophotometer at 560 nm.

Lupane derivatives – 3,4-Seco-lupane type triterpenoids chiisanoside, chiisanogenin, 24-hydroxylchiisanogenin, and 22α-hydroxychiisanogenin are main components of *Acanthopanax divaricatus var. albeofructus* (Family Araliaceae), which have been used in Korea as a tonic, sedative and ginseng-like activities (Oh *et al.*, 2000). Chiisanoside, a main component of this plant, has been reported to have anti-hepatotoxic, antidiabetic and immunostimulant activities. The chiisanoside could be quickly transformed to chiisanogenin by hesperidinase or human intestinal bacteria (Bae *et al.*, 2001). In this study, identification and determination of chiisanoside metabolite was detected by TLC system: stationary phase, silica gel 60F254; solvent, chloroform-methanol-water (65:35:10, lower layer); detection wavelength, 380 nm; coloring sprayer, 10% sulfuric acid and heating. The chromatograms of the developed TLC spots were quantitatively analyzed with a TLC scanner.

Betulinic acid, which is a main component of *Zizyphus vulgaris*, was also determined by TLC (Li, 1986). Betulinic acid glycosides such as betulinic acid-3-O-glucopyranoside identified to be transformed to betulinic acid by intestinal bacteria using the above TLC system (unpublished data).

Oleanane derivatives – GL is a main component of licorice extract (*Glycyrrhiza glabra*), which is frequently used as a herbal medicine. By the oral administration of GL to human, GA was detected in the sera, but GL was not (Nakano *et al.*, 1980). Hattori *et al.* (1983, 1985) reported that GL is transformed to GA by human intestinal microflora. Thereafter, GA was metabolized to 3-epi-18α-glycyrrhetic acid (EGA) *via* 3-dehydro-18α-glycyrrhetic acid (DGA). These metabolites were identified by TLC (Merk silica gel 60F254) system: They used acetic acid-n-butanol-1,2-dichloroethanol-water (4:1:4:1) for separation of GL, GA-monoglucuronide and GA, chloroform-petroleum ether-acetic acid (5:5:1) for separation of GA, EGA, and DGA. The chromatogram of the developed TLC was quantitatively analyzed with a TLC scanner (λs 250 nm; λr 400 nm).

In assay system of GL-metabolic enzyme activity of intestinal bacteria, Akao et al (1987, 1988) used benzene-dioxane-acetic acid (75:20:2) for separation of steroids and bile acids on TLC, and chloroform-petroleum ether-acetic acid (5:5:1) for separation of GA metabolites. Metabolites on TLC plates were detected under UV light or by spraying with 10% sulfuric acid and then heating for 5 min. The GA metabolite was detected on TLC plate under UV light. The quantity was analyzed with a TLC scanner (λs 250 nm; λr 400 nm). By the analytic methods, Akao *et al.* (1987) reported that GL was directly metabolized to GA by *Eubacterium* GLH, a human intestinal bacterium. Kim *et al.* (1996, 1999, 2000) determined metabolites of GL by intestinal bacteria *Eubacterium* L-8 and *Streptococcus* LJ-22. To determine metabolites of GL-administered rats, Akao *et al.* (1987) also used the same analytic method. When the activity of GL-metabolizing β-glucuronidase was assayed, they also used the same mobile phase and detector.

Kalopanaxsaponins are main components of the stem bark of *Kalopanax pictus* (Family Araliaceae), which has

been used as tonic, analgesic and antidiabetic agents in Korea (Sano *et al.*, 1991; Shao *et al.*, 1989; Lee and Hahn, 1991). Its main components are hederagenin glycosides (kalopanaxsaponin A-J). These compounds could not be easily absorbed from intestinal tract to the blood when they were orally administered. Kalopanxsaponin (KP) A isolated as a metabolite showed more potent inhibitory effect on rheumatoid arthritis induced by FCA in rats than KPI (Kim *et al.*, 2002). However, intraperitoneal administeration of KPK was not effective.

Actually when KPK was incubated with human intestinal bacteria, that KPH, KPJ, and KPI were quickly produced and then KPA and hederagenin were slowly produced was identified by TLC (Kim *et al.*, 1998 and 2002). They used chloroform-methanol-water (65:35:10, lower layer) as a mobile phase, 5% sulfuric acid in alcohol as a coloring agent and TLC scanner (detection wavelength, 380 nm) as a detector.

Soyasaponins, which are olean-12ene triterpenes with a C28 methyl group and a glucuronic acid moiety linked at the C3 of the triterpene, are main saponins present of *Glycine max* (Family Leguminosae) (Evans, 2002). The soyasaponins have been reported to show anticarcinogenic, hepatoprotective, and hypocholesterolemic effects. Identifying and isolating these saponins are too difficult. Nevertheless TLC has been used as an analytic method for seperation of these saponins soyasapogenol A, A1, A2 and B, and soyasaponin I, II, and III. Gurfinkel and Rao (2002) used chloroform-methanol-water (65:35:10, lower layer) system as a developing solvent, 10% sulfuric acid in methanol and heating for 15 min at 120°C. For measurement of desitometry, model 620 Biorad Video Densitometer was used in reflectance mode.

To elucidate the metabolism and bioavailability of dietary soyasaponins in human gastrointestinal, Hu and Zeng (2003) investignated metabolism of soyasaponin I and the metabolites were analyzed by TLC and HPLC. They identified that the soysaponin I metabolized soyasaponin III and soyasapogenol B.

Saikosaponins are main components in rhizome of *Bupleurum falcatum*, and Shibata *et al.* (1966) analyzed by TLC using chloroform-methanol (3:1) as a mobile phase. However, this mobile phase could not separate saikosaponins well. Fujiwara and Ogihara (1986) orally administered saikosapnin a to rats, and analyzed its metabolites (prosaikogenin F and saikogenin F) in blood by TLC system. The blood was extracted with chloroform-methanol-water (65:35:10, lower layer) and evaporated in vacuo and analyzed by TLC using the same solvent as a mobile phase. By the analytic methods, Shimizu *et al.* (1985) identified that saikosaponin a was transformed into 2 structural isomers by incubation with rat gastric juice, and that the saikosaponins were changed into monoglycoside and algycones by anaerobic incubation with mouse intestinal microflora *in vitro*. They also identified that saikosaponin c and d are also similarly transformed to that of saikosaponin a. *In vivo* metabolic study, they identified saikosaponin a, its 2 isomers and their metabolites for blood sample.

Yen *et al.* (1991) assayed saikosaponins a, c, and d in Xiao-chai-hu-tang, a herbal medicinal formula, of which ingredients are 8 g of Root of *Bupleurum* spp., 5 g of Tuber of *Pinellia ternata*, 3 g of Root of *Scutellaria baicalensis*, 3 g of Root of *Panax ginseng*, 3 g of fruit of *Zizyphus vulgaricus*, 2 g of Root of *Glycyrrhiza glabra* and 1 g of Rhizome *Zingiber officinale* by TLC-scanner system. They extracted this formula with methanol, and centrifuged it. The supernatant was then evaporated, dissolved in methanol, and spotted in TLC plate. They used ethylacetate-ethanol-water (8:2:1) as a mobile phase, and Kieselgel 60 F254 (Merck, precoated) as a TLC plate. The plates were sprayed with 15% sulfuric acid and heated for 3 min at 105°C. The colored plate was detected by a CAMAG TLC scanner. They detected in less than 2 μg with good recovery. They suggested that this method rapid and useful and rapid for determination of saikosaponins in herbal formula.

Ursane derivatives – Ursolic acid is a representative ursane compound, and Guo *et al.* (1995) determined the content of ursolic acid in folium of *Ilicis cornutae*, *Crataegus pinnatifida*) was determined by TLC-densitometer (TLC scanner, Shimazu CS-920, Japan). To analyze the ursolic acid in *Saliva officinalis* L. leaves, Baricevic *et al.* (2001) extracted it with chloroform extract, filtrated with active carbon, added water, centrifuged and the precipitate was spotted to TLC by means of the Linomat IV spotter (Camag, Muttenz, Switzerland) and analyzed using silica gel 60 HPTLC plates (10 × 20 cm) with or without fluorexent indicator (Merk, Germany). The mobile phase consisted of benzene, ethylacetate and formic acid (36:12:5). The plates were detected after spraying the plated with anisaldehyde-sulfuric acid reagent or molybdophosphoric acid reagent and observing the plates at 254, 366, or 560 nm. In the present review the studies on analysis and identification of herbal medicine components and their metabolites by HPTLC except ursolic acid in *Saliva officinalis* are not introduced. 3-O-Glucopyranosyl ursolic acid was identified to be transformed to ursolic acid by human intestinal bacteria using TLC-densitometry system

(Silica gel 60F254; mobile phase, benzene-ethyl acetate-formic acid (36:12:5) and chloroform-methanol-water (65:35:10, lower layer); coloring agent, 5% sulfuric acid in alcohol; detection wavelength, 380 nm) (unpublished data).

Conclusion

TLC has been used successfully in the separation and determination of a large number of flavonoids and saponins in herbal medicine extracts and their metabolites. TLC is also the most suitable technique for surveying properties of herbal medicine components and their metabolites. In a large number of investigations, the flavonoids and saponins have been analyzed qualitatively and quantitatively with TLC-densitometry or TLC-colorimetry.

Extraction is the first important step for determination of herbal medicine components and their metabolites by TLC. In order to extract the metabolites from urines or culture fluids of intestinal microflora, fecal microflora, most frequently liquid-liquid phase extraction (if necessary, solid-phase extraction on cartridges) has been used. The techniques of solvent extraction are mostly based on the correct choice of solvents. The sample preparation must be correctly selected before sample spotting onto the TLC system. Most frequently silica gel plates (silica gel 60F254, GF254 or IB2F) have been used as a stationary phase. At first, chloroform-acetic acid-water and benzene-dioxane-acetic acid for the analysis of phenolic acids metabolized from flavonoids, chloroform-methanol-water and chloroform-methanol for those of aglycones metabolized from flavonoids, and chloroform-methanol-water and chloroform-ethyl acetate-methanol-water for those of the metabolites of saponins, have been considered as developing systems (Table 1). To qualitatively or quantitatively determine the spots in developed TLC plates, TLC-densitometry, which is a slit-scanning densitometry, is the dominant method of recording thin layer separations for interpretation and quantification. This technology is now relatively mature, and, although limited to absorption and fluorescence detection in the UV-visible range, has adequately served the needs of TLC for the last two decades. Major improvements may be realized through image analysis, also known as video densitometry. The attractions of video densitometry for detection in TLC are fast and simultaneous data acquisition from the whole plate. Flavonoids and their metabolites were generally detected under UV light. If not detectable, the most frequently used visualization sprayers include iodine vapors and 10% sulfuric acid in alcohol. However, most saponin and their metabolites except glycyrrhizin could not be detected under UV light. To detect saponins and their metabolites, sulfuric acid in alcohol and p-anisaldehyde-sulfuric acid in alcohol have been frequently used as visualization sprayers. Based on our experiences, linear relationship between the peak area and the amount of standard phenolic metabolites of flavonoids can be found in the range of 0.5 – 5 μg per spot with recovery being at the level of 95% and standard deviations (SD) of around 5%. Those of flavonoid and their glycosides can be generally detected in the range of 0.1 – 5 μg at 98% and SD of about 3 – 5%. Those of saponins and their metabolites can be found in the range of 0.7 – 5 μg per spot with recovery being at the level of 98% and SD of around 3 – 5%. Most reports did not demonstrate the recovery. Therefore, TLC is highly recommended in metabolic studies of herbal medicine components. Because TLC can simultaneously determine large numbers of samples and do not require any tedious clean-up steps prior to analysis. The TLC technique can be applied for determination of metabolites of herbal medicine extracts as well as herbal medicine formulae.

Flavonoids, saponins and their metabolites can be determined colorimetrically in the crude extract and TLC is just a means of confirmation of their presence in the sample, or the TLC-separated band are scraped, extracted with appropriate solvent, treated with a specific reagent and determined by colorimetry. However, a major problem with TLC techniques is first of all the parallel running of the appropriate standards minimizing the variation between different plates and color reactions with spraying agents. The second difficulty is spot detection by means of sophisticated instrumentation for data acquisition. This can be achieved, however, by on-line coupling of a computer. A great number of flavonoids, saponins and their metabolites are being determined by these techniques.

TLC are a very useful technique for qualitative and quantitative analyses of flavonoids, saponins and their metabolites, and published data will give some hopes for future use of this technique in routine metabolite analysis.

Acknowledgement

I thank for technical assistances of Dr. Bae EA, who is research associate in College of Pharmacy, Kyung Hee Unversity.

References

Abe N., Ebina T., and Ishida N., Interferon induction by

glycyrrhizin and glycyrretinic acid in mice. *Microbiol. Immunol.*, **26**, 535-539 (1982)

Ahmed F., Vyas V., Saleem A., Li X.G., Zamek R., Cornfield A., Haluska P., Ibrahim N., Rubin E.H., and Gupta E., High-performance liquid chromatographic quantitation of total and lactone 20(S)-camptothecin in patients receiving oral 20(S)-camptothecin. *J. Chromatogr. B Biomed. Sci. Appl.* **707**, 227-233 (1998).

Akao T., Akao T., and Kobashi K., Purification and characterization of 7beta-hydorxysteroid dehydrogenase from Ruminococcus sp of human intestine. *J. Biochem.* **102**, 613-619 (1987).

Akao T., Akao T., Hattori M., Namba T., and Kobashi K., Purification and properties of 3alpha-hydroxyglycyrrhetinate dehydrogenase of *Clostridium innocuum* form human intestine. *J. Biochem.* **103**, 504-507 (1988).

Akao T., Kida H., Kanaoka M., Hattori M., and Kobashi K., Intestinal bacterial hydrolysis is required for the appearance of compound K in rat plasma after oral administration of ginsenoside Rb1 from Panax ginseng. *J. Pharm. Pharmacol.*, **50**, 1155-1160 (1998).

Akao T. Kawabata K., Yanagisawa E., Ishihara K., Mizuhara Y., Wakui Y., Sakashita Y., and Kobashi K., Baicalin, the predominant flavone glucuronide of scutellariae radix, is absorbed from the rat gastrointestinal tract as the aglycone and restored to its original form. *J. Pharm. Pharmacol.*, **52**, 1563-1568 (2000).

Baccou J.C., Lambert F., and Sauvaire Y., Spectrophotometric method for the determination of total steroidal sapogenin. *Analyst* **102**, 458-465 (1977).

Bae E.A., Han M.J., Lee K.T., Choi J.W., Park H.J., and Kim D.-H., Metabolism of 6"-O-xylosyltectoridin and tectoridin by human intestinal bacteria and their hypoglycemic and in vitro cytotoxic activities. *Biol. Pharm. Bull.* **22**, 1314-1318 (1999).

Bae E.A., Park S.-Y., and Kim D.-H., Constitutive β-glucosidases hydrolyzing ginsenoside Rb1 and Rb2 from human intestinal bacteria. *Biol. Pharm. Bull.*, **23**, 1481-1485 (2000).

Bae E.A., Yook C.S., Oh O.J., Chang S.Y., Nohara T., and Kim D.-H., Metabolism of chiisanoside from Acanthopanax divaricatus var. albeofructus by human intestinal bacteria and its relation to some biological activities. *Biol. Pharm. Bull.* **24**, 582-585 (2001).

Bae E.-A., Han M.J., Choo M.-K., Park S.-Y., and Kim D.-H., Metabolism of 20(*S*)- and 20(*R*)-ginsenoside Rg3 by human intestinal bacteria and its relation to *in vitro* biological activities. *Biol. Pharm. Bull.*, **24**, 58-63 (2002).

Baricevic D., Sosa S., Loggia R.D., Tubaro A., Simonovska B., Krasna A., and Zupancic A., Topical anti-inflammatory activity of *Salvia officinalis* L. leaves: the relevance of ursolic acid. *J. Ethnopharmacol.* **75**, 125-132 (2001).

Bokkenheuser V.D., Shackleton H.L., and Winter J., Hydrolysis of dietary flavonoid glycosides by strains of intestinal *Bacteroides* from humans. *Biochem. J.*, **248**, 953-957 (1987)

Booth A.N., Murry C.W., Jones F.T., and DeEds F., The metabolic fate of rutin and quercetin in the animal body. *J. Biol. Chem.*, **223**, 251-258 (1956).

Bosland M.C., McCormick D.L., Melamed J., Walden P.D., Zeleniuch-Jacquotte A., and Lumey L.H., Chemoprevention strategies for prostate cancer. *Eur. J. Cancer Prev.* **11**, Suppl 2 S18-27 (2002).

Chen Y.C., Shen S.C., Chen L.G., Lee T.J., and Yang L.L., Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. *Biochem. Pharmacol.* **61**, 1417-1427 (2001).

Cheng K.-J., Krishnamurty H.G., Jones G.A., and Simpson F.J., Identification of products produced by the anaerobic degradation of naringin by Butyrivibrio sp. C3. *Can. J. Microbiol.*, **17**, 129-131 (1971).

Choo M.K., Park E.K., Han M.J., and Kim D.-H., Antiallergic activity of ginseng and its ginsenosides. *Planta Med.* **69**, 518-522 (2003).

Conn J.W., Rovner D.R., and Cohen E.L., Licorice induced pseudoaldosteronism, hypertentsion, hypolalemia, aldosteronopenia and supressed plasma renin activity. *J. Am. Med. Ass.*, **205**, 80-84 (1968).

Corthout J., Naessens T., Apers S., and Vlietinck A.J., Quantitative determination of ginsenosides from Panax ginseng roots and ginseng preparations by thin layer chromatography-densitometry. *J. Pharm. Biomed. Anal.*, **21**, 187-192 (1999).

Dong A., Ye M., Guo H., Zheng J., and Guo D., Microbial transformation of ginsenoside Rb1 by Rhizopus stolonifer and Curvularia lunata. *Biotechnol. Lett.* **25**, 339-344 (2003).

Evans W.C., Trease and Evans Pharmacognosy (15th ed) Saunders press, **214**, 289 (2002).

Finney R.S.H. and Somers G.F., The anti-inflammatory activity of glycyrretinic acid and derivatives. *J. Pharm. Pharmacol.*, **10**, 613-620 (1958).

Francis G., Kerem Z., Makkar H.P., and Becker K., The biological action of saponins in animal systems: a review. *Br. J. Nutr.* **88**, 587-605 (2002).

Fujita H., Sakurai T., and Toyoshima S., Studies on the regulation by drugs against experimental hepatitis (1) The therapeutical effects of glycyrrhizinic acid, DL-methionine, their complex and tablets against acute injuly induced by carbon tetrachloride or D-galactosamine. *Oyo Yakuri*, **16**, 637-645 (1978) (in japanese).

Fujiwara K. and Ogihara Y., Pharmacological effects of oral saikosapnin may differ depending on conditions of the gastrointestinal tract. *Life Sci.*, **39**, 297-301 (1986)

Griffiths L.A., Studies on metabolism of flavonoids. *Biochem. J.*, **92**, 173-175 (1964).

Griffith L.A. and Barrow A., Metabolism of compounds in germ-free rats, *Biochem. J.*, **130**, 1161 (1972a).

Griffith L.A. and Smith G.E., Metabolism of myricetin and related compounds in the rat metabolite formation *in vivo* and by the intestinal microflora *in vitro*. *Biochem. J.* **130**, 141-151 (1972b).

Griffiths L.A. and Smith G.E., Metabolism of apigenin and

related compound in the rat. *Biochem. J.* **128**, 901-911 (1972c).

Guo Y., Cui Y., Zhou J., and Yua P. n, Quantitative determination of ursolic acid in folium Ilicis cornutae (gouguye) gathered in different and periods. *Zhongguo Zhong Yao Za Zhi.* **20**, 591-592 (1995).

Gurfinkel D.M. and Rao A.V., Determination of saponins in Legumes by direct densitometry. *J. Agric. Food Chem.*, **50**, 426-430 (2002).

Han B.H., Park M.H., Han Y.N., Woo W.S., Sankawa U., Yahara S., and Tanaka O., Degradation of ginseng saponins under mild acidic conditions. *Planta Med.*, **44**, 146-149 (1982).

Han Y.O., Han M.J., Park S.H., and Kim D.-H., Protective effects of kakkalide from Flos puerariae on ethanol-induced lethality and hepatic injury are dependent on its biotransformation by human intestinal microflora. *J. Pharmacol. Sci.* **93**, 331-336 (2003).

Hasegawa H., Sung J.-H, Matsumiya S., and Uchiyama M., Main ginseng saponin metabolites formed by intestinal bacteria. *Planta Med.*, **62**, 453-457 (1996).

Hasegawa H., Lee K.S., Nagaoka T., Tezuka Y., Uchiyama M., Kadota S., and Saiki I., Pharmacokinetics of ginsenoside deglycosylated by intestinal bacteria and its transformation to biologically active fatty acid esters. *Biol. Pharm. Bull.* **23**, 298-304 (2000).

Hattori M., Sakamoto T., Kobashi K., and Namba T., Metabolism of glycyrrhizin by human intestinal bacteria. *Planta Med.*, **48**, 38-42 (1983).

Hattori M., Shu Y.Z., Shimizu M., Hayashi T., Morita N., Kobashi K., Xu G.J., and Namba T., Metabolism of paeoniflorin and related compounds by human intestinal bacteria. *Chem. Pharm. Bull.*, **33**, 3838-3846 (1985).

Hattori M., Sakamoto T., Yamagishi T., Sakamoto K., Konishi K., Kobashi K., and Namba T., Metabolism of glycyrrhizin by human intestinal bacteria flora. Isolation and characterization of human intestinal bacteria capable of metabolizing glycrhrhzin and related compounds. *Chem. Pharm Bull.*, **33**, 210-217 (1985).

Hayakawa J., Noda N., Yamada S., and Uno K., Studies on physical and chemical quality evaluation of crude drugs preparations. I. Analysis of Puerariae Radix and species Puerariae. *Yakugaku Zasshi,* **104**, 50-56 (1984).

Hsu H.Y., Chen Y.P., and Hong M., The Chemical Constituents of Oriental Herbs. Oriental Healing Arts Institute press, 1982, 374, 435, 480, 528, 536, 845.

Hu J. and Zheng Y., Metabolism of soyasapnin I by human intestinal microflora in vitro. The 225th ACS national meeting, AGFD 88 (2003).

Jost W. and Hauc H. E. k, The use of modified silica gels in TLC and HPTLC. *Adv. Chromatagr.* **27**, 129-165 (1987).

Kanaoka M., Akao T., and Kobashi K., Metabolism of ginseng saponins, ginsenosides, by human intestinal flora. *J. Med. Pharm. Soc. Wakan-Yaku* **11**, 241-245 (1994).

Kanaoka M., Akao T., and Kobashi K., Appearance of compound K, a major metabolite of ginsenoside Rb1 by intestinal bacteria, in rat plasma after oral administration - measurement of compound K by enzyme immunoassay. *Biol. Pharm. Bull.*, **21**, 245-249 (1998).

Kanaoka M., Akao T., and Kobashi K., Metabolism of ginseng saponins, ginsenosides, by human intestinal bacteria. *J. Tradit. Med.*, **11**, 241-245 (1998).

Karikura M., Miyase T., Tanizawa H., Taniyama T., Takino Y., and Hayashi T., Studies on absorption, distribution, excretion and metabolism of ginseng saponins. V. The decomposition products of ginsenoside Rb2 in the large intestine of rats. *Chem. Pharm. Bull.* **39**, 2859-2861 (1990).

Keinan-Boker L., van Der Schouw Y.Y., Grobbee D.E., and Peeters P.H., Dietary phytoestrogens and breast cancer risk. *Am. J. Clin. Nutr.* **79**, 282-2828 (2004).

Kim D.H., Herbal medicines are activated by intestinal microflora. *Nat. Prod. Sci.,* **8**, 35-43 (2002).

Kim D.-H., Jang I.-S, Kim N.-J., and Youn W.-K., Metabolism of poncirin and naringin by human intestinal bacteria. *Yakhak Hoeji*, **38**, 286-292 (1994)

Kim D.H., Jang I.S., Lee H.K., Jung E.A., and Lee K.Y., Metabolism of glycyrrhizin and baicalin by human intestinal bacteria. *Arch. Pharm. Res.*, **19**, 292-296 (1996).

Kim D.H., Han S.B., Bae E.A., and Han M.J., Intestinal bacterial metabolism of rutin and its relation to mutagenesis. *Arch. Pharm. Res.*, **19**, 41-45 (1996).

Kim D.H., Jung E.A., Sohng I.S., Han J.A., Kim T.H., and Han M.J., Intestinal bacterial metabolism of flavonoids and its relation to some biological activities. *Arch, Pharm. Res.*, **21**, 17-23 (1998).

Kim D.-H., Kim N.J., Bae E.A., and Han M.J., Scientific analysis of Daekumeumja and Pyungwesan by the metabolism of hesperidin. *Kor. J. Pharmacogn.* **29**, 136-141 (1998).

Kim D.-H., Yu K.-U., Bae E.A., and Han M.J., Metabolism of puerarin and daidzin by human intestinal bacteria and their relation to in vitro cytotoxicity. *Biol. Pharm. Bull.* **21**, 628-630 (1998).

Kim D.-H., Yu K.W., Bae E.A., Park H.J., and Choi J.W., Metabolism of kalopanaxsaponin B an H by human intestinal bacteria and antidiabetic activity of their metabolites. *Biol. Pharm. Bull.*, **21**, 360-365 (1998).

Kim D.H., Lee S.W., and Han M.J., Biotransformation of glycyrrhizin to 18β-glycyrrhetinic acid 3-O-β-D-glucuronide by *Streptococcus* LJ-22, a human intestinal bacterium. *Biol. Pharm. Bull.*, **22**, 320-322 (1999).

Kim D.-H., Kim S.-Y., Park S.-Y., and Han M.J., Metabolism of quercitrin by human intestinal bacteria and its relation to some biological activities. *Biol. Pharm. Bull.* **22**, 749-751 (1999).

Kim D.H., Hong S.W., Kim B.T., Bae E.A., Park H.Y., and Han M.J., Biotransformation of glycyrrhizin by human intestinal bacteria and its relation to biological activities. *Arch. Pharm. Res.*, **23**, 172-177 (2000).

Kim D.H., Bae E.A., Han M.J., Park H.J., and Choi J.W., Metabolism of kalopanaxsaponin K by human intestinal bacteria and antirheumatoid arthritis activity of their

metabolites. *Biol. Pharm. Bull.* **25**, 68-71 (2002).

Kirshmanmury H.G., Cheng K.J., Janes G.A., Simpson F.J., and Watkin J.E., Identification of products produced by the anaerobic degradation of rutin and related flavonoids by Butyrivibrio sp. C3. *Can. J. Microbiol.* **16**, 759-767 (1970).

Knekt P., Kumpulainen J., Jarvinen R., Rissanen H., Heiovaara M., Reunanen A., Hakulinen T., and Aromaa A., Flavonoid intake and risk of chronic diseases. *Am. J. Clin, Nutr.* **76**, 560-568 (2002).

Kobashi K. and Akao T., Relation of intestinal bacteria to pharmacological effects of glycosides. *Bifidobacteria Microflora* **16**, 1-7 (1997).

Kumagai A., Yano S., Otome M., and Takeuchi K., Study on the corticoid-like action of glycyrrhizinic and the mechanism of its action. *Endocrinol. Jpn.* **4**, 12-27 (1957).

Kuroki G. and Poulton J.E., The para-O-methylation of apigenin to acacetin by cell-free extracts of Robinia pseudoacacia L., *Z. Naturforsch.* **36c**, 916-920 (1981).

Kurita A. and Kaneda N., High-performance liquid chromatographic method for the simultaneous determination of the camptothecin derivative irinotecan hydrochloride, CPT-11, and its metabolites SN-38 and SN-38 glucuronide in rat plasma with a fully automated on-line solid-phase extraction system, PROSPEKT. *J. Chromatogr. B Biomed. Sci. Appl.* **724**, 335-344 (1999).

Lee J.M., Longevity and life Preservation in Oriental Medicine (In: S.H. Choi, translated), Kyung Hee Univ. Press, Seoul, pp.153 (1996).

Lee M. and Hahn D.R., Triterpenoidal saponins from the leaves of *Kalopanax pictum* var. *chinense*. *Arch. Pharm. Res.*, **14**, 124-128 (1991).

Lee S.J., Sung J.H., Lee S.J., Moon C.K., and Lee B.H., Antitumor activity of a novel ginseng saponin metabolite in human pulmonary adenocarcinoma cells resistant to cisplatin. *Cancer Lett.*, **144**, 3943 (1999).

Lee D.S., Kim Y.S., Ko C.N., Cho K.H., Bae H.S., Lee K.S., Kim J.J., Park E.K., and Kim D.H., Fecal metabolic activities of herbal components to bioactive compounds. *Arch. Pharm. Res.*, **25**, 165-169 (2002).

Li S.Y., Quantitative determination of betulinic acid in Zizyphus vulgaris by TLC-colorimetry. *Zhong Yao Tong Bao* **11**, 43-45 (1986).

Li Y.F. and Zhang R., Reversed-phase high-performance liquid chromatography method for the simultaneous quantitation of the lactone and carboxylate forms of the novel natural product anticancer agent 10-hydroxycamptothecin in biological fluids and tissues. *J. Chromatogr. B Biomed. Sci. Appl.* **686**, 257-265 (1996).

Li B.Q., Fu T., Dongyan Y., Mikovits J.A., Ruscetti F.W. and Wang J.M., Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry. *Biochem. Biophys. Res. Commun.* **276**, 534-538 (2000).

Liu C.X., Development of Chinese medicine based on pharmacology and therapeutics. *J. Ethnopharmacol.* **19**, 119-123 (1987).

Liu G., Gu F., Xu K., and Zhang L., Research on the transformation of ginsenoside Rg1 by intestinal flora. *Zhongguo Zhong Yao Za Zhi* **26**, 188-190 (2001).

Liu A.P., Fang Z.Q., and Huang X., Consideration for the study of basic theory of TCM. *Zhongguo Zhong Xi Yi Jie He Za Zhi.* **22**, 249-254 (2002).

MacDonald I.A., Mader J.A., and Bussard R.G., The role of rutin and quercitrin in stimulating flavonol glycosidase activity by cultured cell-free microbial preparation human feces and saliva. *Mutation Res.* **122**, 95-102 (1983).

MacGregor J.R., Mutagenicity studies of flavonols in vivo and in vitro. *Toxic. Appl. Pharm.* **48**, A47 (1979).

Manach C., Morand C., Texier O., Favier M.L., Aullo G., Demigne C., Regerat F., and Remesy C., Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *J. Nutr.* **125**, 1911-1922 (1995).

Manach C., Morand C., Demigne C., Texier O., Regerat F., and Remesy C., Bioavailability of rutin and quercetin in rats. *FEBS Lett.* **409**, 12-16 (1977).

Maugh T.H., TLC: The overlooked alternative. *Science* **216**, 161-163 (1982).

Middleton E., Kandaswami C., and Theoharides T.C., The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Rev.* **52**, 673-751 (2000).

Mitsuoka T., A color atlas of anaerobic bacteria, Sobunsa, Tokyo, 322 (1980).

Morton M.S., Arisaka O., Miyake N., Morgan L.D., and Evans B.A., Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. *J. Nutr.* **132**, 3168-3171 (2002).

Nakano N., Kano H., Suzuki H., Nakano N., Yano S., and Kanako M., Enzyme immunoassay of glycyrrhetic acid and glycyrrhizin 2. Measurement of plasma glycyrrhetic acid and glycyrrhizin *Jpn. Pharmacol. Ther.* **8**, 4171-4174 (1980) (in japanese).

Nohara T. and Kinjo J., Chemical constituents of Puerariae Radix. *J. Tradit. Sino-Jpn Med.* **13**, 391-397 (1984).

Oh O.J., Chang S.Y., Yook C.S., Yang K.S., Park S.Y., and Nohara T., Two 3,4-seco-lupane triterpenes from leaves of Acanthopanax divaricatus var. albeofructus. *Chem. Pharm. Bull.* **48**, 879-881 (2000).

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

Pompeo R., Flore O., Marccialis M.A., Pani A., and Loddo B., Glycyrrhizic acid inhibits virus growth and inactivates virus particles. *Nature* **281**, 689-690 (1979).

Poole C.F., Planar chromatography at the turn of the century. *J. Chromatogr.* **A 856**, 399-427 (1999).

Poole C.F., Thin-layer chromatography: challenges and opportunities. *J. Chromatogr.* **A 1000**, 963-984 (2003).

Poole C.F. and Poole S.K., Progress in densitometry for quantitation in planar chromatography. *J. Chromatogr.* **492**, 539-584 (1989).

Poole C.F. and Dias N.C., Practitioner's guide to method development in thin-layer chromatography. *J. Chromatogr.* **A 892**, 124-142 (2000).

Prosek M., Golc-Wondra A., and Vovk I., Uncertainty in quantitative thin-layer chromatography. *J. Chromatogr. Sci.* **40**, 598-602 (2002).

Rechner A.R., Kuhnle G., Bremner P., Hubbard G.P., Moore K.P., and Rice-Evans C.A., The metabolic fate of dietary polyphenols in humans. *Free Rad. Biol. Med.* **33**, 220-235 (2002).

Sano K., Sanada S., Ida Y., and Shoji J., Studies on the constituents of the stem bark of *Kalopanax pictus*. *Chem. Pharm. Bull.* **39**, 865-870 (1991).

Shao C.J., Kasai R., Xu J.D., and Tanaka O., Saponins from roots of *Kalopanax septemlobus* (Thunb.) Koidz., Ciqiu: Structures of kalopanaxsaponins C, D, E and F. *Chem. Pharm. Bull.* **37**, 311-314 (1989).

Shibata S., Kitagawa I., and Fujimoto H., The chemical studies on oriental plant drugs. XV. On the constituents of Bupleurum spp. (2). The structure of saikogenin a, a sapogenin of Bupleurum falcatum L., *Chem. Pharm. Bull.* **14**, 1023-1033 (1966).

Shibata S., Tanka O., Ando T., Sado M., Tsushima S., and Ohasawa T., Chemical studies on oriental plant drugs. XIV. Protopanaxadiol, a genuine sapogenin of ginseng saponins. *Chem. Pharm. Bull.* **14**, 595-600 (1966).

Shim S.B., Kim N.J., and Kim D.H., β-Glucuronidase inhibitory activity and hepatoprotective effect of 18-glycyrrhetinic acid from the rhizomes of *Glycyrrhiza uralensis*. *Planta Med.* **65**, 40-43 (2000).

Shimizu K., Amagaya S., and Ogihara Y., Structural transformation of saikosaponins by gastric juice and intestinal flora. *J. Pharmacobio-Dyn.* **8**, 718-725 (1985).

Stoewsand G.S., Anderson J.L., Boyd J.N., and Harzdine G., Quercetin: A Mutagen not a carcinogen in fischer rats. *J. Toxic. Environ. Health.* **14**, 105-114 (1984).

Tamura G., Gold C., Ferro-Luzzi A., and Ames B.N., Fecalase-A model for activation of dietary glycosides to mutagens by intestinal flora. *Proc. Natl. Acad. Sci.* in USA, **77**, 4981-4965 (1980).

Tangri K.K., Seth P.K., Parmar S.S., and Bhargava K.P., Biochemical study of anti-inflammatory and antiarthritic properties of glycyrretic acid. *Biochem. Pharmacol.*, **14**, 1277-1281 (1965).

Tsai T.H., Analytical approaches for traditional chinese medicines exhibiting antineoplastic activity. *J. Chromatogr. B Biomed. Sci. Appl.* **764**, 27-48 (2001).

Wakabayashi C., Hasegawa H., Murata J., and Saiki I., *In vivo* antimetastatic action of ginseng protopanaxadiol saponins is based on their intestinal bacterial metabolites after oral administration. *Oncol. Res.* **9**, 411-417 (1998).

Winter J., Moore L.H., Dowell V.R., and Bokkenheuser V.D., C-ring cleavage of flavonoids by human intestinal bacteria. *Appl. Environ. Microbiol.* **55**, 1203-1208 (1989).

Wu J.Y., Gardner B.H., Murphy C.I., Seals J.R., Kensil C.R., Recchia J., Beltz G.A., Newman G.W., and Newman M.J., Saponin adjuvant enhancement of antigen-specific immune responses to an experimental HIV-1 vaccine. *J. Immunol.* **148**, 1519-1525 (1992).

Wu J., Lin L., and Cha F. u, Ultrasound-assisted extraction of ginseng saponins from ginseng roots and cultured ginseng cells. *Ultrasonics Sonochemistry* **8**, 347-352 (2001).

Yen M.-H., Lin C.-C, Chuang C.-H., and Liu S.-Y., Evaluation of root quality of Bupleurum species by TLC scanner and the liver protective effects of Xiao-chai-hu-tang prepared using three different Bupleurum species. *J. Ethnopharmacol.* **34**, 155-165 (1991).

Yip Y.Y., Lau C.N.B., But P.P.H., and Kong Y.C., Quantitative analysis of ginsenosides in fresh Panax ginseng. *Am. J. Chinese Med.* **8**, 77-88 (1985).

Youn W.K., Kim D.H., Kim N.J., and Hong N.D., Biological active components of fruits of *Poncirus trifoliata*. *Yakhak Hoeji* **36**, 548-555 (1992).

Zhang C., Yu H., Bao Y., An L., and Jin F., Purification and characterization of ginsenoside-beta-glucosidase from ginseng *Chem. Pharm. Bull.* **49**, 795-798 (2001).

Zheng C., Ji L., Chen G., Chen J., and Tang Z., Quality standard of tanre kesou granules. *Zhongguo Zhong Yao Za Zhi* **21**, 348-350 (1996).

Zhu Y.P., Chinese Materia Medica, Harwood academic publishers, 1-43 (1988).

(Accepted August 21, 2004)