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IDENTIFICATION AND CHARACTERIZATION OF PLANT EXTRACT PHARMACEUTICALS USING VIBRATIONAL SPECTROSCOPIC TECHNIQUES

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Abstract:

This paper reports the possibility of monitoring the sweetener content in the leaves of Stevia Rebaudiana and root extract of Ginseng using FT-IR, micro-Raman and SERS techniques. Fingerprint analysis is a direct, fast, nondestructive and effective method to discriminate and identify different species. Ginseng root contains ginsengoside saponin about 4% and other active compounds such as D-glucose, D-fructose, sucrose, maltose, trisaccharide A,B,C, cellulose, alkaloids, volatile oil, etc. Stevioside is a natural sweet-tasting glycoside isolated from the herb Stevia rebaudiana, composed of stevia, a diterpenic carboxylic alcohol with three glucose molecules, mainly used commercially as sugar substitute. Stevioside, comprising 6-18% of the stevia leaf, is also the most prevalent glycoside in the leaf and is considered 300 times sweeter than sucrose at 0.4% sucrose concentration, 150 times sweeter at 4% sucrose, and 100 times sweeter at 10% sucrose concentration. Other sweet constituents include steviolbioside, rebausiosides A-E, and dulcoside A. Vibrational analysis of Stevia Rebaudiana reveals the specific modes of the three glucose molecules and the diterpenoic carboxylic alcohol with the characteristic Raman bands at 1663, 1198, 1126, 746 cm^{-1}. Regarding the ginseng, the starch content is monitored through the band intensity at 1026 cm^{-1}. The peaks at 911, 856 and 766 cm^{-1} are assigned as characteristic absorption of the carbohydrate I, II and III whereas the week band at 990 cm^{-1} in the secondary derivative FT-IR is closely related to the content of saponin. It is well known that the content of saponin in wild American ginseng may reach 11%. The sensitivity of the FT-vibrational techniques allowed identifying the differences due to the cultivation origin and it can be applied to determine the composition of a plant mixture. Such analysis may be applied to test the authenticity of particular products and ensure their proper use. The results provide a useful tool in preventing the fraudulent substitution of one type of plant for another.

Keywords: *S*tevia rebaudiana, Ginseng, FT-IR, Raman spectroscopy, SERS

INTRODUCTION

The experimental developments have demonstrated that surface enhanced Raman scattering (SERS) together with the FT-vibrational techniques is a potential tool to provide a platform for the development of portable sensing devices for environmental, industrial or biomedical purpose [1]. In conjunction with the continuously developed field of the nanosystems, SERS sensing turns the focus area for the interdisciplinary research. Combining SERS methods with the recent developments in the highly ordered metallic nanosurfaces, one can obtain relevant information for *in-vivo* measurements, with distinct advantages over the expensive and time consuming H-NMR or HPLC methods.

Stevia rebaudiana Bertoni, a plant originating from Paraguay is a safe, all-natural alternative to artificial sweeteners and refined sugar in the diet due to the natural sweeteners contents, stevioside and rebaudioside A. The sweetener started to be used in sugar-free versions of diet food products. Recent links between stevia extract and the normalization of blood sugar and insulin in diabetics were developed, besides the applications used in herbal medicines and cosmetic products. As a table top sweetener the stevioside has no calories, no carbohydrates, no fat and is 300 times sweeter than sugar. Stevia leaves as well as pure stevioside extract (fig.1) can be processed in the food industry without altering its molecular species in different pH media, high temperatures (up to 200 degrees), etc. In spite of its enormous economical importance, only a few preliminary studies were reported [2-5].

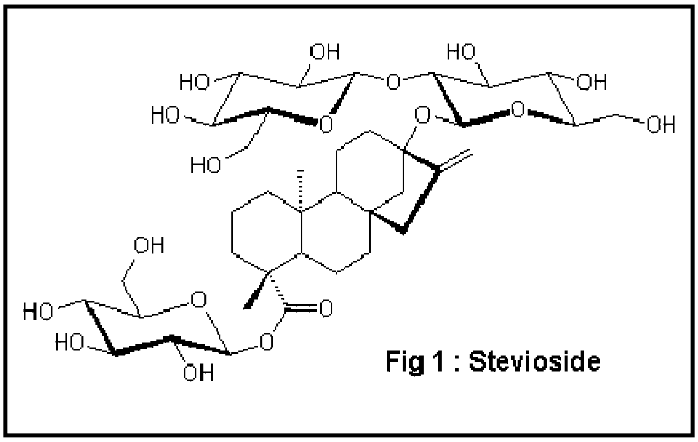

Fig. 1 The molecular structure of stevioside, a natural sweet-tasting glycoside isolated from the herb Stevia rebaudiana, composed of steviol, a diterpenic carboxylic alcohol with three glucose molecules, mainly used commercially as sugar substitute.

This paper reports the possibility of monitoring the sweetener content in the leaves of Stevia Rebaudiana using micro-Raman and surface enhanced Raman techniques. In order to apply the SERS sensing to the herb biological development, the vibrational analysis of the stevioside and the other steviol-related glucosides contained in the leaves were performed. Different distinct regions of the crop leaves were analysed using SERS. The stevioside deposits in the specific leaves sections were evidenced.

Ginseng is one of the most popular products of the Traditional Chinese Medicines and is used widely in the western world as well as the Far East. Its activity depends on the species grown and on the part of the plant root that is processed, so it is important to be able to distinguish between them. A new type of authentication process involved uses the recent technique of proteomics as well as FR-IR and Raman spectroscopy. Ginsengosides are the main active constituents of ginseng. The root, root-stock, stems, leaves, flowers and flower-buds of the ginseng plant contain more than 30 ginsengosides [6-11].The accepted nomenclature of the individual saponins named Rx (x=o, a, b1, b2, c, d, e, f, g1, g2 …) is based on the sequence of spots detected after silica gel thin layer chromatography. Ginsengosides are tri-terpenoid glycosides of the dammaran series. They can be divided into three types according to the characteristics of their chemical structure: oleanolic acid, panaxadiol and panaxtriol types. The chemical structures of these ginsengosides have been detected by IR, MS, NMR and chemical reactions [7]. Ginsengosides are highly glycosylated and their activity often differs depending on the number of glycosyl units attached [7,8]. It exhibits central nervous system-depressant and antipsychotic activity, protection of stress ulcer, increase of gastrointestinal motility and weak anti-inflammatory action [7,10,11]. Chemical structures of these ginsengoside are presented in Fig.2.

Pattern recognition techniques combined with rapid and non-destructive analytical instrumentation have already attracted considerable attention for the purpose of classification or discrimination. Especially both near infrared (NIR) and Raman spectroscopy, which can be generally applied with no or minimal sample preparation, have been extensively investigated for qualitative analysis for the purpose of classification. Our paper reveals new possibility of monitoring the ginsengosides content of ginseng root (ginsengoside saponin about 4%) and other active compounds such as D-glucose, D-fructose, sucrose, maltose, trisaccharide A,B,C, cellulose, alkaloids, volatile oil, etc.

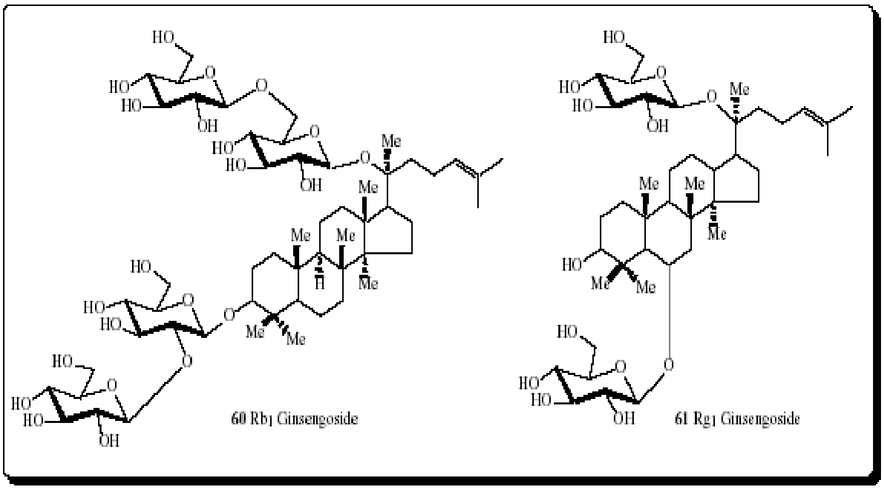

Fig.2 The molecular structure of two different ginsengoside

EXPERIMENTAL

The Stevia Rebaudiana plants were obtained from the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania and the pure powder stevioside from the commercial sources (Fluka). Commercial Ginseng powder used as soluble tea from China has been analyzed using FT-IR and FT Raman spectroscopy. FT-IR spectrum was recorded in the region 4000-650 cm^{-1} using a Bruker EQUINOX 55 spectrometer with an Attenuated Total Reflectance accessory. The internal reflection element was a ZnSe ATR plate (50 x 20 x 2 mm) with an aperture angle of 45°.

A micro-Raman setup (Ramanscope II) connected with optical fiber to the FRA 106 S Raman module was employed in order to record the FT Raman spectrum. The 1064 nm line of a Nd:YAG laser was applied for excitation. An InGaAs detector was used. The laser power was 50 mW, the exposure time 1000 s and 40 overlaps were collected. The spectral resolution was 2.0 cm^{-1}.

The citrate reduced Ag colloid was used as SERS substrate and a micro-Raman set-up of the Dylor LabRam integrated system with the 514.5 nm excitation line was employed for the measurements. The sections of the fresh cutted plants were immersed into the colloid and then fixed on the microscopic plate for Raman allignment. Three overlaps / 2 min. were set for the spectra collection. A Peltier cooled CCD detection was performed with a resolution of 2 cm-1.

RESULTS AND DISCUSSIONS

Stevia Rebaudiana.

Raman and SERS spectra of stevioside have been recorded and discussed. Vibrational analysis provided the specific vibrational modes of the three glucose molecules and the diterpenoidic carboxylic alcohol (Fig. 3) with the characteristic Raman bands at 1663, 1198, 1126, 746 cm-1. In addition to stevioside, the Stevia leaves (fig.4) contain several kinds of the steviol-related glucosides, like steviolbioside, rebaudioside A, the latter being a steviol-tetraglucoside, known as the main sweet constituents after stevioside.

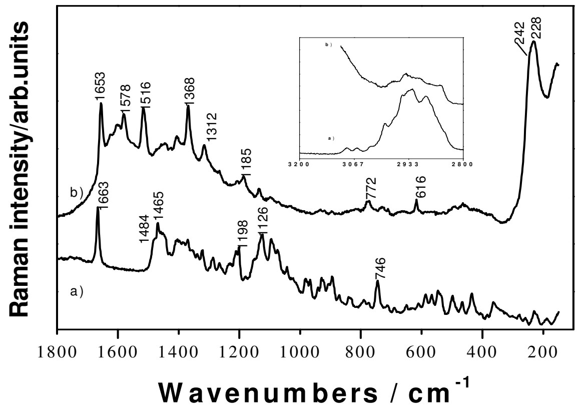

Fig.3. Raman a) and SERS b) spectra of the stevioside. Excitation: 514.5 nm, 50 mW.

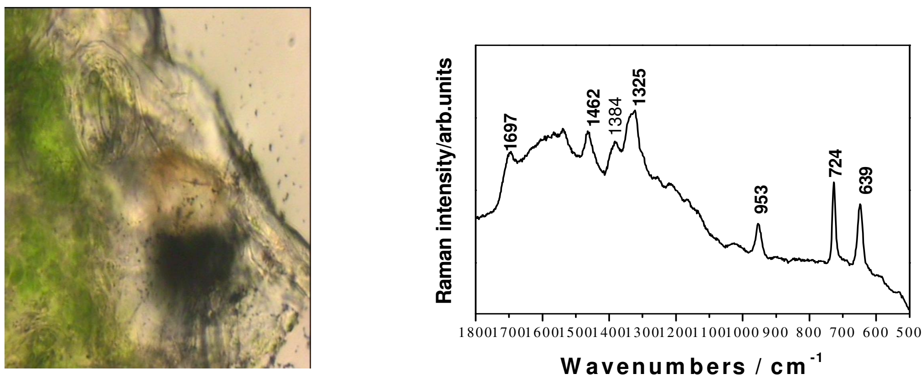

Fig.4. Microscopic image of a Stevia plant section with high stevioside content (left) and its corresponding SERS spectrum (right).

A concentration dependence SERS study was also performed. The relative intensities of the SERS spectra (Fig. 5) were unchanged, revealing a stable conformation to adsorption of the stevioside through the diterpene carboxylic moiety on the Ag particles. Very different SERS behaviour was observed for the stem at different hiigh and at different biological stage as well. The young fresh leaf revealed poor SERS signal whereas the leafs from the bottom of the stem present distinct regions with stevioside accumulation. Any signal from dulcoside was completely absent at the rooth level.

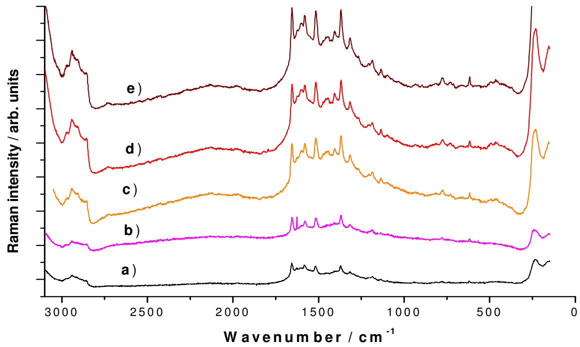

Fig.5. Concentration dependence SERS spectra of the stevioside: a) 0.5, b)1, c) 1.5, d) 2, e) 2,5 x 10-3 moll-1. Excitation: 514.5 nm, 50 mW.

Chinese Ginseng.

The characteristic vibrational modes of ginsengoside are due to oleanolic acid saponin, panaxadiol and panaxatriol saponins. The FT-IR spectrum (Fig. 6a) shows their macro fingerprint features. For example the band at 3309 cm^{-1} is representative for C-H stretch, the band at 2923 cm^{-1} is due to the stretching vibration of $–CH_2$- groups, the 1633 cm^{-1} line is due to the stretching vibration of carbonyl group in the volatile oils and other compounds containing C=O group [12]. Many C-O-C groups exhibit characteristic bands in the 1150 - 911 cm^{-1} spectral range and generally the strong peak at 1026 cm^{-1} is assigned to the vibration of C-O in alcohol hydroxyl group. Compared with other recent studies in this field [6, 12-14], the bands at 1056-1026 cm^{-1} can be assigned as characteristic modes for distinguishing different types of ginseng (from America, Korea or China). The strong peak at 1026 cm^{-1} in our FT-IR spectrum is also an indicative of the starch content in the sample: the stronger the relative intensity of the band, the higher the starch content. This fact may be also proved by the second derivative spectrum (fig.7). The peaks at 911, 856 and 766 cm^{-1} are assigned as characteristic absorption of the carbohydrate according to reference [13], respectively to carbohydrate I, II and III. The FT-IR spectrum of the previously reported ginseng species [12] contains also some characteristic bands of calcium oxalate from 1317 and 782 cm^{-1}. The relative intensity of these bands (as well as the carbohydrate's peaks) is small in our FT-IR spectrum, revealing the low content of oxalates. The Raman spectrum of ginseng powder (Fig. 6b) exhibits the more accurate and well resolved bands than the IR one. However, the strong overlapping contributions make difficult to differentiate between sensitive related species.

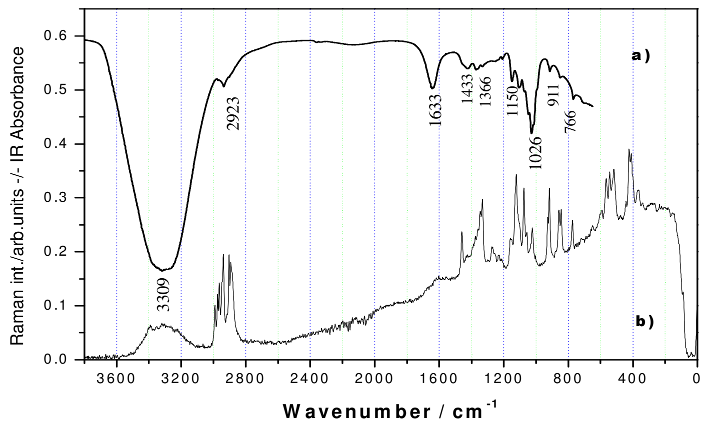

Fig. 6. a) FT-IR and b) FT Raman spectra of powder Ginseng from China.

Strong and well resolved bands in the FT-Raman spectrum (Fig. 6b) indicate the presence of the starch and carbohydrates and less the corresponding oxalate stretching. Another interesting feature is the week band at 995 cm^{-1} present in the Raman spectrum and at 990 cm^{-1} in the secondary derivative FT-IR, closely related to the content of saponin [6, 12, 15]. It is well known that the content of saponin in wild American ginseng may reach 11% [12]. So, different types of ginseng can be distinguished from each others taking into account the relative intensity of this band in the FT Raman spectrum [11, 12,16].

The SERS spectra in this case was not relevant, but using the advantage of the secondary derivative spectrum by the apparent enhancing of the spectral resolution, we amplify the tiny differences in the 1600-800 cm^{-1} spectral range for both the FT-IR and FT-Raman spectra. The secondary derivative IR and Raman spectra of ginseng powder are presented in the Figs. 7 and 8 respectively. Taking a closer examination of the observed bands, based on comparison with the Korean, Beijing and American ginseng species [12], we concluded a Chinese species as dominant in our spectra. The dominant fingerprints were attributed to the carbohydrates and less to starch and oxalates. Therefore, these molecular species is more related to the Beijing and Korean ginseng than to the American versions.

As our sample has a commercial purpose as tea, we have investigated its behavior in diluted and saturated aqueous solution. Fig.9 presents the detailed Raman spectrum of the solid sample compared to the spectra of the dissolved sample (diluted and saturated) in the 700-1800 cm^{-1}spectral range, where the characteristic fingerprints are displayed.

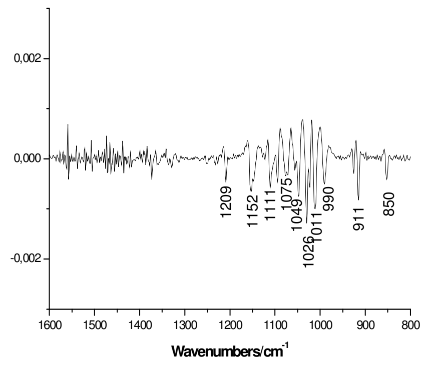

Fig. 7. Secondary derivative IR spectrum of ginseng powder.

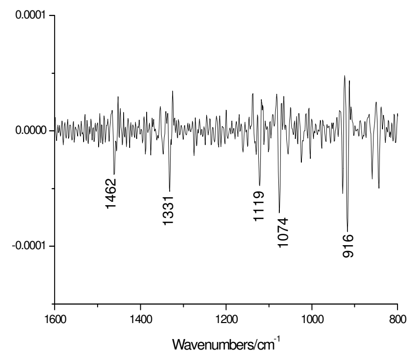

Fig. 8. Secondary derivative FT-Raman spectrum of ginseng powder.

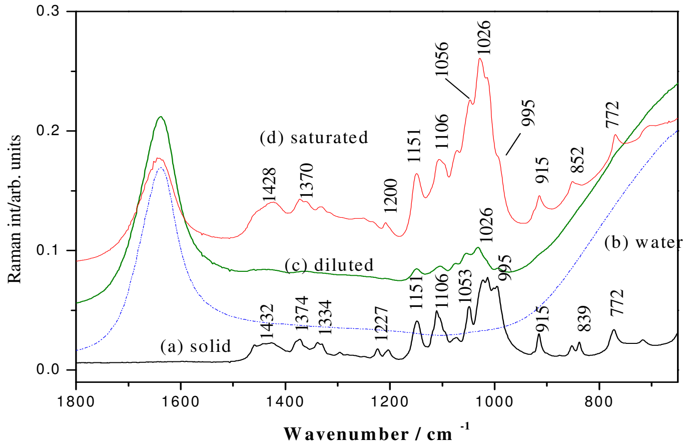

Fig.9. FT-Raman spectra of a) powder ginseng , b) water, c) dilluted solution and d)saturated solution of ginseng. Excitation: 1064 nm, 50 mW.

One can observed that the spectral features are very well preserved in the saturated solution and the relative intensity of the bands in the range 950-1320 cm^{-1} similar to those from Raman spectrum of the powder. So, the starch content monitored through the band intensity at 1026 cm^{-1} is favourable to be measured in saturated solution.

CONCLUSIONS

The stevioside sweetener was characterised using Raman and SERS spectroscopy. The molecule was found to adsorb on the Ag colloidal surface through the diterpene carboxylic moiety. A stabile conformation to adsorption was observed in the concentration dependence SERS study.The SERS analysis illustrates the leaves domain with high stevioside content. Quantitative and time dependence SERS study *in vivo* allowed monitoring the stevia crops development.

The sensitivity of the FT-vibrational techniques allowed identifying the various parts of the ginseng root or the differences due to the cultivation origin and it can be applied to determine the composition of a ginseng mixture. Such analysis may be applied to test the authenticity of particular products and ensure their proper use. The results provide a useful tool in preventing the fraudulent substitution of one type of ginseng for another.

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Classification of cultivation area of ginseng radix with NIR and Raman spectroscopy

The Analyst

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A rapid and non-destructive method for the classification of ginseng radix according to cultivation area (Korea and China) was evaluated using near infrared (NIR) reflectance spectroscopy. Ginseng samples were collected from widely different growing areas of Korea and China to give more geographical variations. Although there were no obvious differences in the raw NIR spectra based on cultivation area, the spectral features were enhanced and differentiated by utilizing a second-derivative algorithm. Using principal component analysis the cultivation area was clearly differentiated in the principal component space. To develop a classification rule, partial least squares discriminant analysis was carried out. The origin of ginseng was successfully classified and predicted using NIR reflectance spectroscopy combined with discriminant analysis. Additionally, FT-Raman spectroscopy, which provides more qualitative information and easy sample measurement, was also investigated for the same purpose. The results showed that the classification performance using NIR reflectance spectroscopy was superior to that using Raman spectroscopy.

Ginseng, the root of *Panax ginseng* C. A. Meyer, has been considered as an important component of many traditional prescriptions in Korea.[1] It is now a well known herbal medicine throughout the world and is mainly employed for the purpose of health care in Asia, North America and Europe. The action of ginseng is generally known to increase arousal, stamina and resistance to stress.[2,3] As the use of ginseng has become very popular, it is being cultivated in many areas. The efficacy and quality of ginseng differ widely according to the growing conditions, especially the cultivation area (Korea, China), although it comes from the same species. Unfortunately, it is difficult to classify ginseng according to cultivation area with existing analytical techniques and it has been dependent on visual inspection. Therefore, there is an increasing demand for a reliable and fast analytical method to determine the cultivation area for the proper evaluation and distribution of the product.

Pattern recognition techniques combined with rapid and non-destructive analytical instrumentation have already attracted considerable attention for the purpose of classification or discrimination.[4] Especially both near infrared (NIR) and Raman spectroscopy, which can be generally applied with no or minimal sample preparation, have been extensively investigated for qualitative analysis for the purpose of classification. NIR pattern recognition methods have been widely applied to the classification of vegetable oil[5] and roasted coffees.[6] It has recently been reported that Raman spectroscopy and chemometrics have been applied to the discrimination of ivories.[7] Additionally, chemometric classification using pyrolysis mass spectrometry has been attempted to discriminate cocoa butter[8] and some European wines.[9] These researches were performed only with the mass spectra, without regard to chemical components.

In the present study, pattern recognition techniques using NIR reflectance and FT-Raman spectroscopy were investigated to develop a practical classification method. Principal component analysis (PCA) was utilized to ascertain the possibility of classification in principal component (PC) spaces and, with partial least squares (PLS) discriminant analysis,[10,11] was used to develop a classification method. Because ginseng is a very complex mixture, as are other herbal medicines, the individual NIR and Raman spectra were similar to each other. However, the spectral features of ginseng in specific regions were slightly different from each other and these spectral differences resulted in the successful classification of ginseng based on its origin. The classification performance of NIR was better than that of Raman spectroscopy, even though the Raman spectra have sharper features. This is because the reproducibility of Raman spectra is degraded owing to both the sample matrix and fluorescence. Additionally, the signal-to-noise ratio of the Raman spectrum was poor compared with the NIR spectrum.

Experimental

Sample preparation

All ginseng radix samples (roots of *Panax ginseng* C. A. Meyer) were acquired from the Experimental Station of the Natural Agriculture Products Inspection Office (NAPIO), Seoul, Korea. Fifty Korean and 50 Chinese ginseng samples were collected. Each ginseng sample was collected from widely different growing areas of Korea (Kyunggi, Kanghwa, Kyungbuk, *etc.*) and China (Jilin) to give more geographical variations. All the samples were dried when acquired. All samples were powdered using a cyclone mill fitted with a 1 mm screen. The particle size of powder was controlled below 20 mesh.

NIR reflectance spectra

Visible–NIR reflectance spectra were collected over the 400–2500 nm spectral region with a NIRSystems Model 6500 spectrometer (Foss NIRSystems, Silver Spring, MD, USA) equipped with a quartz halogen lamp, silicon detector (visible region) and PbS detector (NIR region). The spectra were collected at 2 nm intervals. The spectra were acquired with a circular sample cup with a quartz window (38 mm in diameter and 10 mm in thickness). Each sample spectrum was obtained