Quantitative Prediction of Agarotetrol in Chinese Eaglewood Using Near Infrared Spectroscopy

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To overcome the numerous disadvantages of existing testing technology, a novel, fast, nondestructive, and quantitative technology for quality evaluation of Chinese eaglewood (CE) based on near-infrared (NIR) technology was proposed in this study. The extractives of CE were qualitatively analyzed to determine the types of volatile compounds using gas chromatography-mass spectroscopy and were quantitatively determined using high performance liquid chromatography (HPLC). Agarotetrol was quantitatively determined by the HPLC analysis. The content was found to range widely from 0.016 to 0.104 mg/g. A quantitative prediction model aimed at quality control was proposed based on the qualitative and quantitative results coupled with a partial least squares regression. The coefficient of correlation and residual predictive deviation of the prediction model were determined to be 0.9697 and 5.77, respectively. The practical tests showed an average error of 0.000327%, which indicated that the method was able to provide a novel, quick, and effective quality evaluation of CE.

Keywords: Chinese eaglewood; Qualitative and quantitative analysis; NIRS; Prediction model; High-performance liquid chromatography (HPLC)

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INTRODUCTION

Chinese eaglewood (CE) (*Aquilaria sinensis* Lour. Gilg), which is formed by the decomposition of fungi in an anaerobic environment (Jalaluddin 1977; Qi 1995), is widely distributed in southern China, Indonesia, Malaysia, and other tropical areas (Yagura *et al*. 2003; Naef 2011). It has become famous and popular for its versatile uses in sedatives, and its analgesic and digestive properties (Yagura *et al*. 2003; Chen *et al*. 2012). It is also used in prayer beads, religious objects, and incense-burning regimens in traditional Chinese cultures. The amount of wild CE has been dramatically reduced because of the increasing demand for it in the Chinese market over the past two decades. Since 2004, all of the wild *Aquilaria* species have been listed on the Appendix II list of the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora for their protection (Li *et al*. 2016). As a consequence, fake CE products have been extensively produced because of the surging demand and increasing scarcity of CE. In many ways, economic benefits have been achieved through the falsification of other similar wood species, counterfeiting high-quality CE with low-grade CE, or by impregnating wood with chemical compounds that have odors that are similar to the volatile constituents in the extractives of CE. In practice, the volatile compounds should not exceed a content level of

10% in qualitatively identified CE, according to the wood species discrimination conducted by this laboratory each year.

Traditional wood identification techniques primarily use light microscopy to conduct research studies in wood anatomy, which is usually sufficient to identify a CE sample at a species level. However, the qualitative identification of the wood species by the volatile compound contents of CE has been found to be almost unremarkable because of the fact that the value of CE is mainly dependent on the volatile compound content in its extractive. It has been proven that the volatile compounds mainly consist of sesquiterpenes, aromatic compounds, 2-(2-phenylethyl) chromones, and aliphatic compounds in wild *Aquilaria* species, which appear to be unique to CE (Naef *et al*. 2011; Li *et al*. 2013a). Moreover, none of the compounds mentioned above have been found to exist in healthy wild *Aquilaria* trees. To date, over 525 types of volatile compounds have been detected in the extractives of CE. These include 155 types of sesquiterpenoids, 162 types of aromatic compounds, 49 types of 2-(2-phenylethyl) chromones, 62 types of aliphatic compounds, and 97 types of other compounds (Zhao *et al*. 2013). It has proven difficult or impossible to entirely separate these compounds to perform a quality evaluation of CE.

Among these volatile constituents, 2-(2-phenylethyl) chromone derivatives and sesquiterpenes, which are abundant in CE and have biological activities including anti-inflammatory properties (Yang *et al*. 2012), have been used as marker constituents (Li *et al*. 2013a; Zhang *et al*. 2015). These compounds have been qualitatively identified, and are also the main indices for the quality evaluation of CE. The marker constituents of CE have generally been detected by gas chromatography-mass spectroscopy (GC-MS) analysis in the past. For the most part, the characteristic fragment ions of 2-(2-phenylethyl) chromone derivatives or sesquiterpenes can be separated and matched with a MS spectrum in the NIST11 and WILEY275 databases. Several hundred types of volatile compounds have been evaluated, and a reliable GC-MS evaluation method has been developed for the qualitative analysis of CE over the past two decades (Chen *et al*. 2011; Mei *et al*. 2013; Xia *et al*. 2013). However, a GC-MS analysis is a qualitative method, and it is only able to obtain the relative contents of the volatile compounds in CE extractives. Therefore, this method has disadvantages when used to perform quantitative evaluations. Recently, a high-performance liquid chromatography (HPLC) analysis has been proposed to qualitatively analyze the volatile compounds in the extractives from CE (Chen *et al*. 2012; Li *et al*. 2013a). Over the past several years, qualitative and quantitative methods, such as GC, HPLC, and tandem MS, have been developed for CE quality evaluation. However, in practice, these methods require sample extractions and separation of constituents, have been found to be complicated by pretreatments, and are time-consuming and expensive for commercial usage. Therefore, the need still remains for a simple and reliable method for the rapid quality evaluation of CE.

The derivatives of 2-(2-phenylethyl) chromone, particularly the highly oxidized 5,6,7,8-tetrahydro-2-(2-phenylethyl) chromones (TEPECs) are characteristic compounds that only exist in CE, and were used as markers for reliability and quality evaluation before 2015. However, it has been very difficult to obtain a relevant index for these quality evaluations because several hundred types of 2-(2-phenylethyl) chromone derivatives exist in CE. Agarotetrol has been identified as a 2-(2-phenylethyl) chromone derivative existing in CE, and was recently used as a substitute marker constituent for 2-(2-phenylethyl) chromone derivatives for quality evaluation indices following the 2015 edition of the *Chinese Pharmacopeia* (Chinese Pharmacopoeia Commission 2015). It has been found

that agarotetrol has a high content and stability, both in wild and cultured CE. According to previous reports, the characteristic peak of agarotetrol, which was analyzed by HPLC, had a good separation effect with an R greater than 1.5 (R representing the separation ratio of the adjacent peak). Moreover, compared with the various parameters of HPLC analysis, it was found that the separation effect of agarotetrol was more stable than that of other 2-(2-phenylethyl) chromone derivatives (Gu *et al*. 2014; Zhang *et al*. 2015). Therefore, agarotetrol was assigned to be the sole marker for quantity in CE since 2015 (Chinese Pharmacopoeia Commission 2015).

As a novel quality control technology, near-infrared (NIR) spectroscopy has advantages over other analytical techniques. This is because it is a fast, easy to use, and low-cost method (Wu *et al*. 2012; Li *et al*. 2013b). Accordingly, NIR has recently been playing an important role in qualitative analysis. More importantly, NIR technology not only has the ability to perform qualitative analyses, it is also able to perform quantitative evaluations, which suggests that it is a potentially effective tool. In this study, to develop an accurate, quick, and nondestructive quality control technology for CE evaluation, a NIR prediction model was proposed based on the qualitative analyses of volatile compounds in extractives by GC-MS and the quantitative detection by HPLC. Furthermore, the reliability of this model was verified in this study.

EXPERIMENTAL

Sample Preparation

The agarotetrol used in this study had a purity of 98.3%. It was purchased from the National Institute for Food and Drug Control (Beijing, China). HPLC-grade acetonitrile and absolute ethanol were purchased from Beijing Chemical Works (Beijing, China). A standard stock solution was prepared by dissolving 12.5 mg of agarotetrol in 100 mL of absolute ethanol. Then, eight batches of CE samples were purchased from Guangxi Forestry Institution (Nanning, China). The samples were first discriminated using the traditional wood discrimination method for observing the anatomy of wood structures with an optical microscope.

All of the CE samples were crushed to a powder using a cyclone mill, and the powder was screened to pass through a 60-mesh sieve. The dried powder (1 g) was extracted with 10 mL of absolute ethanol using an ultrasonic extraction apparatus (40 kHz, 250 W; Kunshan Ultrasonic instrument Co. Ltd. , Suzhou, China) for 1 h at room temperature. The solvent was continuously replenished in the extraction system because of volatilization loss. The extracts were centrifuged at 12000 rpm for 10 min, and the supernatant was filtered through a 0.22-μm membrane nylon filter to acquire the sample extractions required for the GC-MS and HPLC analyses.

Methods

GC-MS analysis of the extractives

A Thermo Scientific Trace GC Ultra gas chromatography instrument combined with a mass selective detector (ISQ, Thermo Scientific, Waltham, Massachusetts, USA) was utilized for analysis of the extractives. Separation of the samples was performed using a DB-5MS phenyl arylene polymer capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Santa Clara, USA) and was virtually equivalent to (5%-phenyl)-methylpolysiloxane. Then, 1.0 μL of the extractives was injected into the front inlet of a

gas chromatograph, which was operated at 250 °C without split injection. The carrier gas was helium with a flow rate of 1.0 mL/min. The oven program commenced at 60 °C, remained at that temperature for 2 min, increased to 250 °C at a rate of 4 °C/min, and the temperature was held for 20 min. The interface temperature was 280 °C. To obtain the ionization of the compounds by electron impact, an emission current of 70 eV was used. The ion source temperature was 280 °C and the scan range was set from 50 amu to 800 amu. The relative contents of the compounds were determined by normalization, and the compounds were characterized using the NIST11 database.

HPLC analysis of the agarotetrol

The HPLC (1290 Infinity, Agilent Technologies, Santa Clara, USA) was equipped with a G1329B DAD detector, G1311C quaternary pump, G1329B auto-sampler, and G1316A column temperature controller (Agilent Technologies, Santa Clara, USA). It was connected to an Agilent ChemStation running on ChemStation software, and was used for an extractive analysis aimed at the characteristic compound agarotetrol. The chromatographic separations were done on an ODS-3 C18 column (250 mm × 4.6 mm id, 5 μm; Intersil, Kyoto, Japan). The mobile phases were composed of acetonitrile (A) and 0.1% formic acid in water (B). The gradient program was as follows: 0 min to 10 min: 15% to 20% A; 10 min to 19 min: 20% to 23% A; 19 min to 21 min: 23% to 33% A; and 21 min to 39 min: 33% to 95% A. A 10-min pre-equilibration and 10-min post run periods were used between the individual runs with a flow rate of 0.7 mL/min. The injection volume was 10 μL and the DAD detector wavelength was set at 252 nm.

NIR spectrum collection and the quantitative prediction model

A total of 50 oven-dried CE samples were divided into eight groups and milled with a plant crusher to pass through a 60-mesh screen to acquire powder samples. Then, the powder was placed into a transparent bottle, and the NIR diffuse-reflectance spectra information was collected with a Fourier transform-NIR spectrophotometer (MPA; Bruker, Karlsruhe, Germany). Figure S1 (Appendix) shows the NIR spectra over a total spectral range of 12500 cm^{-1} to 3600 cm^{-1}, which were acquired using an integrating sphere that scanned an area with a diameter of approximately 20 mm. The 32 scans of the sample spectra were averaged per scan at a spectral resolution of 8 cm^{-1}, and then the spectra were stored as a log (1/reflectance) spectrum. The partial least square (PLS) modeling of the data was completed using the Bruker software OPUS7.2. The calibration models were constructed using PLS regression with a full cross-validation. Then, by combining the NIR spectra and quantitative analysis results for the characteristic compound, an accurate and nondestructive predictive model based on NIR technology was built for advanced quantitative evaluations.

RESULTS AND DISCUSSION

Chemical Analysis of the Volatile Compounds by GC-MS

GC-MS is a powerful tool that was used in this study for the qualitative research of the volatile constituents of *Aquilaria sinensis*. Figure 1 shows the GC-MS total ion chromatogram that had three obvious peaks, which corresponded to the volatile constituents of the extractives in the CE. They were clearly observed at the retention times of 23.19, 28.08, and 28.69 min.

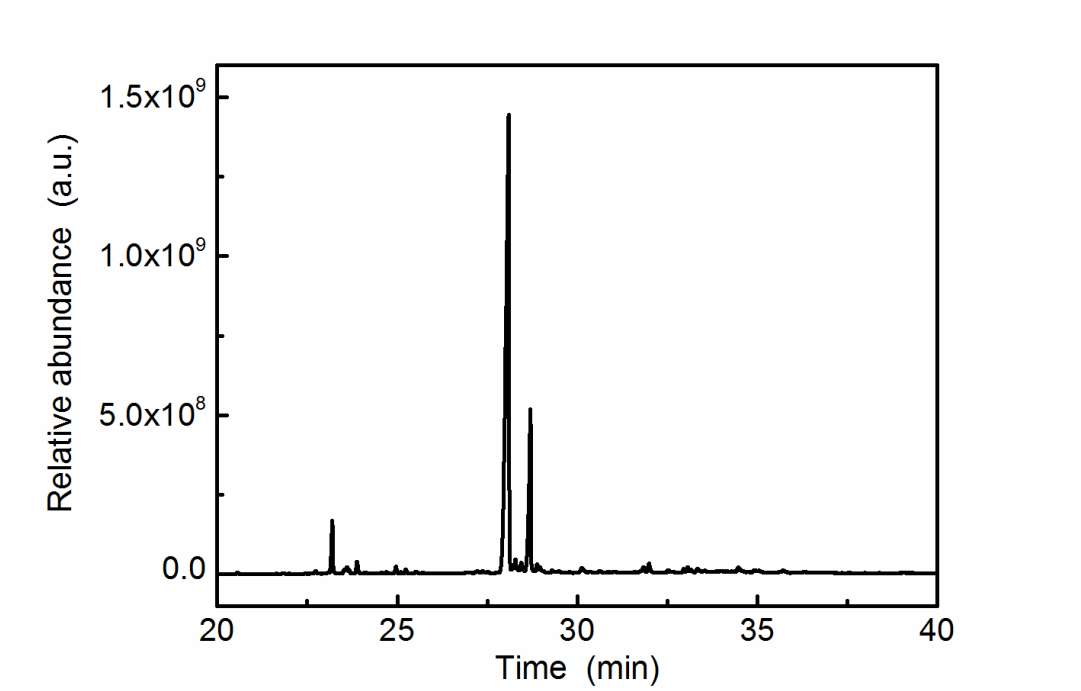

Fig. 1. GC-MS total ion current

Table 1. GC-MS Analysis Results

Peak	Compound	Molecular Formula	RT (min)	Relative Content (%)
1	α-Gurjunene	$C_{15}H_{24}$	22.73	0.31
2	Epi-α-Selinene	$C_{15}H_{24}$	23.19	5.98
3	β-Selinene	$C_{15}H_{24}$	23.6	2.01
4	β-Maaliene	$C_{15}H_{24}$	24.96	0.77
5	Selina-3,7(11)-diene	$C_{15}H_{24}$	25.24	0.46
6	β-Elemol	$C_{15}H_{26}O$	25.52	0.21
7	β-Guaiene	$C_{15}H_{24}$	27.22	0.17
8	β-Vatirenene	$C_{15}H_{22}$	27.37	0.2
9	γ-Eudesmol	$C_{15}H_{26}O$	28.08	48.52
10	8-epi-γ-eudesmol	$C_{15}H_{26}O$	28.28	1.15
11	Aristolene	$C_{15}H_{24}$	28.45	0.87
12	Agarospirol *	$C_{15}H_{26}O$	28.69	17.93
13	Longipinocarveol, trans-	$C_{15}H_{24}O$	28.88	0.84
14	Epiglobulol	$C_{15}H_{26}O$	28.96	0.53
15	Doconexent	$C_{22}H_{32}O_2$	30.13	0.47
16	Isoaromadendrene epoxide	$C_{15}H_{24}O$	30.87	0.25
17	Dihydro-β-agarofuran *	$C_{15}H_{26}O$	31.99	1.84
18	Ledene alcohol	$C_{15}H_{24}O$	34.47	0.51
* Characteristic compound RT: retention time			Total:	83.02%

Table 1 shows the characteristic volatile constituents of *Aquilaria sinensis*, Agarospirol and Dihydro-β-agarofuran, were found at the retention times of 28.69 and 31.99 min with relative abundances of 17.93% and 1.84%, respectively. Two other volatile constituents, Epi-α-Selinene and γ-eudesmol, which are the origin of the special odor of CE, were found at the retentions times of 23.19 and 28.08 min with relative abundances of 5.98% and 48.55%, respectively. The total relative content was determined to be 83.01%, including 18 volatile compounds in the extractives from the CE. These compounds included 17 sesquiterpenes and one aliphatic compound. The sesquiterpene compounds, which were observed to have been present during earlier retention times, were determined to be the dominant components of the CE extractive. The 2-(2-phenylethyl) chromone derivatives, which are the characteristic compounds that express the quality of CE, were not detected by the GC-MS analysis. It was taken into consideration that chromones and their derivatives are usually present after a retention time of 40 min, and are probably also lacking in the mass spectra found in the NIST11 database.

Quantitative Analysis of Agarotetrol Using HPLC

The quantitative analysis of agarotetrol is usually conducted using HPLC. The HPLC spectra of the representative CE extractive sample (solid line), and the agarotetrol standard sample (20 μg/mL) (dotted line) are shown in Fig. 2. The separation peak was found to occur at a retention time of approximately 19.3 min, which demonstrated the presence of agarotetrol in the CE extractives, even though it was not found by the GC-MS analysis performed in this study. The agarotetrol standard samples, which had eight concentration gradients (1, 5, 20, 30, 50, 70, 75, and 100 μg/mL), were analyzed by HPLC under the same testing conditions. Then, according to an external standard method using absolute ethanol as the solvent, the standard curve established to determine the agarotetrol content of the samples was obtained by connecting the peak area and corresponding observed concentration, which is illustrated in Fig. 3.

From the results shown in Fig. 3, it was indicated that the relative coefficient of determination (R^2) of the established standard curve was 0.9998 because of the high linearity from 1 to 100 μg/mL. All of the samples examined in this study showed the same results, which were consistent with the *Chinese Pharmacopeia* (Chinese Pharmacopeia Commission 2015). Furthermore, as shown in Table 2, the content of agarotetrol (average value from 5 to 8 samples) in the eight batches of CE samples were tested. The content levels ranged from 0.016 to 0.104 mg/g, which indicated differences that were nearly 10 times higher. Therefore, the agarotetrol content was found to be very apparent in this study. Similarly, a quantitative evaluation method (Li *et al*. 2013a) of the CE was developed by determining the content of 2-(2-phenylethyl) chromone derivatives in the CE using an HPLC analysis.

The R^2 was determined to be 0.9997 for the eight types of characteristic TEPECs. These results confirmed a good ability and reliability in the determination of the target marker content existing in the CE samples when using HPLC analysis to establish the standard curve. The content of agarotetrol was found to range widely, which extended the testing range and enhanced the adaptive capacity for the detection of unknown samples. This was found to be advantageous for the development of the NIR model based on principal component analyses.

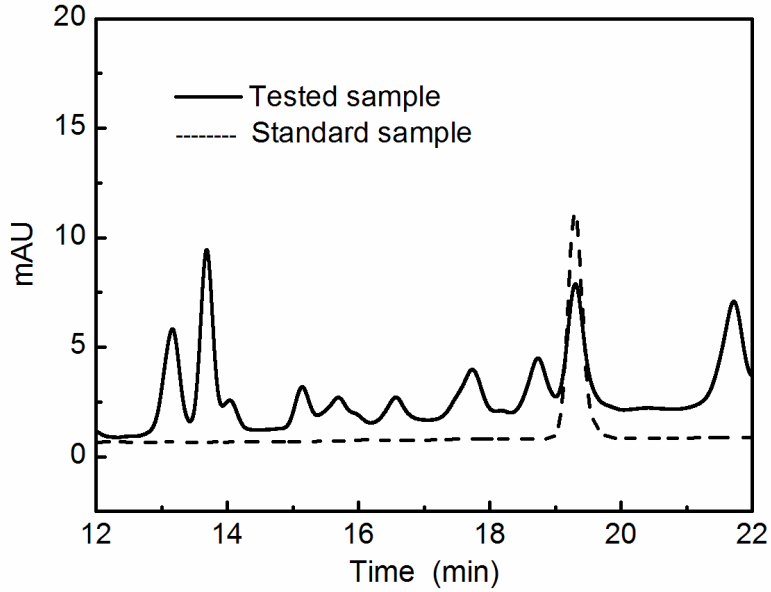

Fig. 2. HPLC spectra of the agarotetrol in the CE extractive (solid line) and agarotetrol standard sample (dotted line) (20 µg/mL)

Table 2. Absolute Content of the Agarotetrol in the CE

Analyte	1	2	3	4	5	6	7	8
Agarotetrol (mg/g)	0.104	0.037	0.027	0.033	0.044	0.03	0.016	0.027

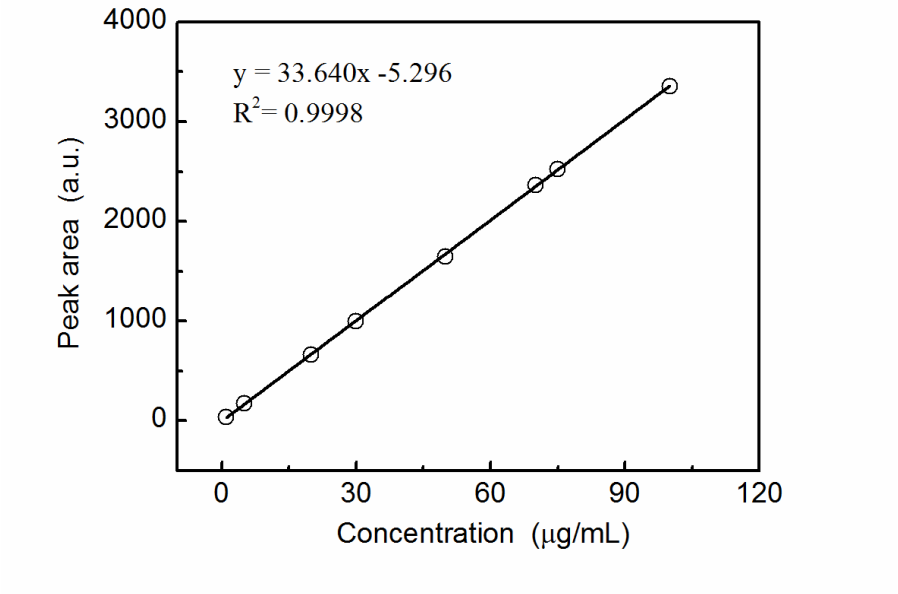

Fig. 3. Standard curve of agarotetrol for eight different concentrations (1, 5, 20, 30, 50, 70, 75, and 100 µg/mL)

NIR Model Development

The representative raw spectra of the CE samples, including the untreated and ethanol-extracted samples, are shown in Fig. 4. The NIR spectra showed a similar trend, except for differences that mainly occurred in the bands at 5195 and 5051 cm^{-1}, which were attributed to the O-H asymmetrical stretching vibration and its deformation vibration of H_2O, respectively (Ali *et al*. 2001; Workman and Weyer 2007), that existed in the samples. A comparison between the second-derivative mode of the NIR spectra of the untreated and ethanol-extracted samples (Fig. 5) showed changes in the -OH with aromatic -CH, $-CH_3$, $-CH_2$, and -CH overtone vibrations.

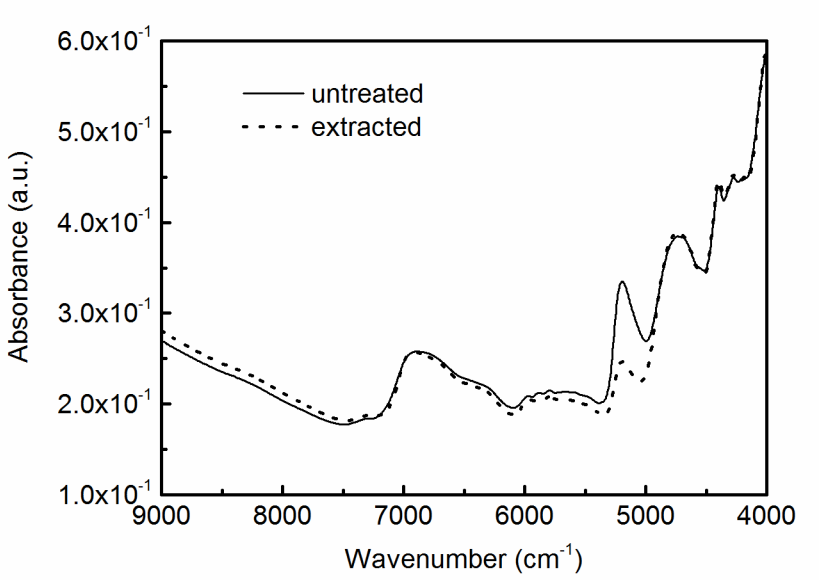

Fig. 4. Raw NIR spectra of untreated and ethanol-extracted CE

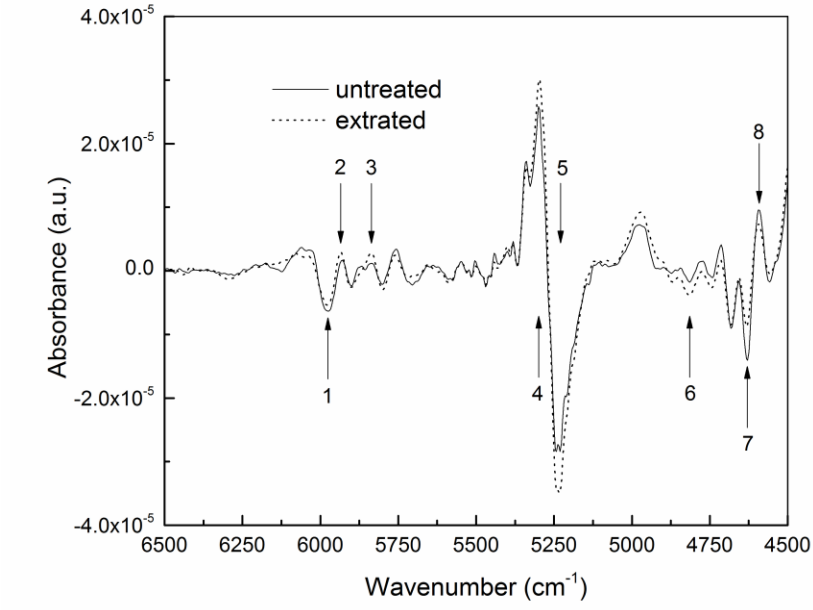

Fig. 5. Second-derivative mode of the NIR spectra of untreated and ethanol-extracted CE

Also, it should be mentioned that the extracts and volatile compounds in the CE, which contained primarily 2-(2-phenylethyl) chromone derivatives and other sesquiterpenes, contained chemical structures of an aromatic ring and C=C. Figure 5 shows that the numbered bands in the derived spectra were part of those already referenced in previous studies. Each was attributed to polymers that are known constituents of wood. Band 1 located at 5976 cm^{-1} was assigned to the C_{ar}-H 1st stretching vibration of aromatic groups in the lignin (aromatic skeletal, or aromatic C-H in lignin) (Workman and Weyer 2007; Tsuchikawa and Siesler 2003; Sandak *et al*. 2011). Band 2 located at 5936 cm^{-1} was assigned to the C_{ar}-H 1st stretching vibration of the aromatic ring associated with aromatic skeletal structures in the lignin (Tsuchikawa and Siesler 2003; Workman and Weyer 2007; Sandak *et al*. 2011). Band 3 located at 5848 cm^{-1} was assigned to the C-H 1st stretching vibration of furanose/pyranose in the hemicellulose (Yonenobu and Tsuchikawa 2003). Band 4 located at 5291 cm^{-1} was assigned to free and weakly H-bonded -OH in the amorphous polysaccharides of the wood (Michell and Schimleck 1996). Band 5 located at 5236 cm^{-1} was assigned to the C=O stretching vibration of the 2nd overtone in the hemicellulose (Siesler *et al*. 2002). Band 6 located at 4811 cm^{-1} was assigned to O-H stretching vibration and C-H deformation vibration in the semi-crystalline or crystalline regions of the cellulose (Siesler *et al*. 2002). Band 7 located at 4686 cm^{-1} was assigned to C_{ar}-H stretching vibration and the C=C stretching vibration in the lignin and extractives (Michell *et al*. 1996). Band 8 located at 4592 cm^{-1} was assigned to cellulose and xylan. These bands showed corresponding absorption peaks, which were mainly free water and -OH groups originating from the hemicellulose and amorphous regions of the cellulose and the phenolic hydroxyl or aromatic groups in the lignin (Schwanninger *et al*. 2011). The locations of the absorption peaks indicated there were differences in the untreated and ethanol-extracted samples. The second-derivative spectra of the untreated wood (solid line) displayed peaks in some regions with stronger intensities. However, the spectra of the ethanol-extracted samples (dotted line) showed less absorption, such as for the bands at 5976 cm^{-1} and 4686 cm^{-1}, which were determined to be closely related to the aromatic ring in the lignin and extractives (Michell *et al*. 1996; Tsuchikawa and Siesler 2003; Workman and Weyer 2007; Sandak *et al*. 2011). Overall, these combined results confirmed that the CE extractives were changed by the extraction pretreatment.

The NIR spectra of the eight CE batches were recorded at room temperature conditions. A total of 45 NIR spectra were combined with the agarotetrol content, which was determined by HPLC, to construct the prediction model. The PLS regression is the statistical method that is most commonly utilized to construct NIR calibration models. A regression method can be used to define the relationship between the spectral data matrix and properties of a material. In this study, a PLS method was combined with different pretreatments to reduce noise, as well as to remove systematic variances caused by scatter. The pretreatments included the first derivative, second derivative, multiple scatter correction, standard normal vector (SNV), and Savitzky-Golay smoothing. The number of window points for the derivatives and smoothing was varied (5, 9, 13, 17, 21, or 25 points) using a first-order polynomial. The pretreatments weakened any problematic baseline shifts or noises in the NIR spectra that may have occurred because of the instrument or samples. A total of 38 samples were exploited to build the calibration models, and a further seven samples were used to validate the model. The populations for the calibration and validation were randomly assigned. A wave-number range was appropriately selected to identify the most effective spectral range for the prediction and to eliminate any prediction errors caused by unnecessary spectral ranges. A cross-validation was employed to optimize

the number of PLS factors and to guide the selection process. Also, mean centering was applied to all of the spectra before performing the variable subset selections and calibrations.

Table 3 shows only the best results from the pretreatment techniques. The R^2 and either root mean square error of the prediction (RMSEP) or calibration (RMSEC) were used to compare the different PLS models of the agarotetrol content. The technique, which combined the PLS regression and selected spectral range, was determined to yield the best calibration model for the agarotetrol content. The R^2 and relative percent deviation (RPD) of the agarotetrol content calibration models were 0.985 and 8.18, respectively. Prediction results are usually considered "excellent" when the R^2 is greater than 0.90 and the RPD is greater than 3; "good" if the R^2 is between 0.81 and 0.90 and the RPD is between 2.5 and 3; "approximate" if the R^2 is between 0.66 and 0.80 and the RPD is between 2.0 and 2.5; or "poor" if the R^2 is less than 0.66 and the RPD is less than 2 (Saeys *et al*. 2005; Jiang *et al*. 2010). The R^2 and RPD of the model were 0.9697 and 5.77 in the validation set, respectively, as shown in Fig. 6 and Table 3, which was considered to be excellent.

The correlation between the measured agarotetrol content values and the corresponding values predicted using the NIR spectra collected over the spectral range of 6102 to 4598 cm^{-1} (1639 to 2175 nm) is shown in Fig. 6. The quality of the best model was based on the values of the R^2, RMSEP, and RPD. The best agarotetrol content cross-validation model used SNV, the first-derivative pretreatment (17 points), and 10 principal components. Using the proposed models, seven samples were randomly selected to test the established technology. The detailed results are illustrated in Table S1 (Appendix). According to the practical tests, these results satisfactorily met the quantitative prediction of the CE with a maximum error of 0.00143% (less than 0.002%) and an average error of 0.000327% (less than 0.0004%), which confirmed that the model proposed in this study successfully predicted the agarotetrol content in the examined CE samples.

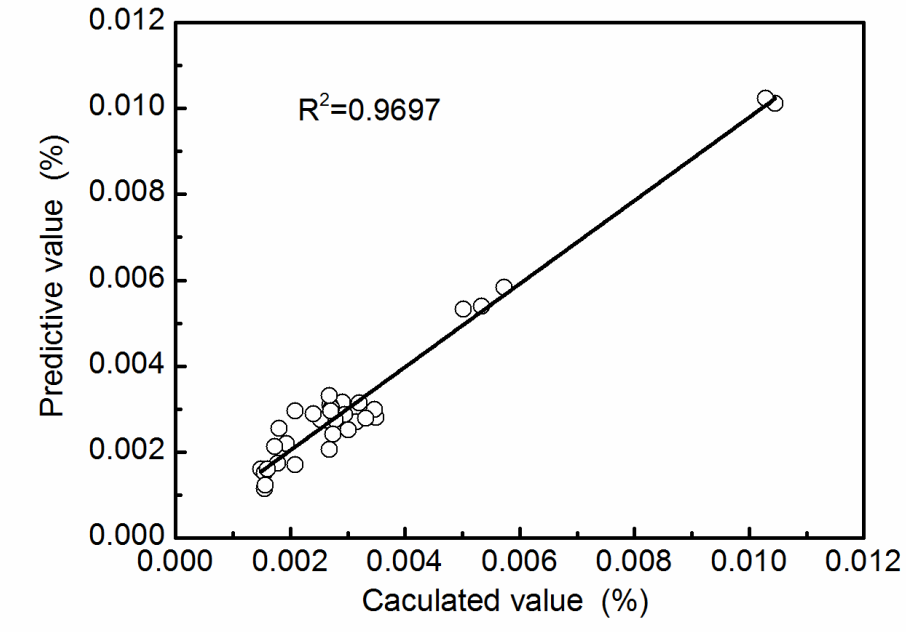

Fig. 6. NIR predictive model of the agarotetrol content

Table 3. Correlation between the NIR Model and Agarotetrol Content in the CE

Style		Agarotetrol Content (%)
Calibration set	R^2	98.5
	RMSEC	0.000289
	RPD	8.18
Validation set	R^2	96.97
	RMSEP	0.00035
	RPD	5.77

CONCLUSIONS

1. In this research, the volatile compounds in the extractives of the CE were qualitatively analyzed by GC-MS. In total, 18 types of volatile compounds, with a total relative content of 83.01%, were detected within a retention time of 40 min by the GC-MS analysis. These compounds included 17 sesquiterpenes and one aliphatic compound.
2. Agarotetrol was one of the 2-(2-phenylethyl) chromone compounds that was quantitatively determined by the HPLC analysis. Its content was found to range widely from 0.016 to 0.104 mg/g.
3. A NIR prediction model, which used agarotetrol as the quality marker for the CE, was successfully constructed in this study. The prediction model, with an R^2 and RPD of 0.9697 and 5.77, respectively, was found to be satisfactory with regards to the quantitative prediction.
4. Applied in a practical test, the constructed NIR prediction model showed a good accuracy with a maximum error of 0.00143% (less than 0.002%) and an average error of 0.000327% (less than 0.0004%), which confirmed that the technology proposed in this study successfully tested the agarotetrol content in the examined CE samples.

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