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Rapid selection of catechin-rich tea trees (*Camellia sinensis*) by a colorimetric method

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Abstract A rapid and efficient colorimetric method based on the use of Fast Blue B-salt (FBB) was established to select catechin-rich tea trees (*Camellia sinensis* L.). The catechin levels measured by the colorimetric method under optimized reaction conditions correlated closely with estimations by high-performance liquid chromatography (HPLC) analysis. The FBB colorimetric method was successfully used to classify 160 tea trees on the basis of their catechin contents into rich and poor lines. HPLC analysis of the FBB-selected tea tree extracts showed them to contain (–)-epigallocatechin 186 mg/g in tea tree line HR-29, (–)-epicatechin 43.7 mg/g in HR-82, (–)-epigallocatechin gallate 4.32 mg/g in HR-29, and (–)-epicatechin gallate 0.22 mg/g in HR-52. Classification of tea trees from the Hadong region into catechin-rich and -poor trees was independent of the growing season. Thus the FBB colorimetric method could find application as a reliable tool in screening and selection of tea trees on the basis of their catechin content.

Key words Selection · Tea tree · Catechin · Fast Blue B salt · Colorimetric method

Introduction

Tea tree (*Camellia sinensis* L.) extracts comprise polysaccharides; flavonoids; vitamins B, C, and E; *R*-amino butyric acid; caffeine; catechin compounds; and fluoride.[1] Catechins form a complex group of compounds. They are made up of (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), (–)-epicatechin (EC), (+)-catechin (C), catechin gallate (CG), (+)-gallocatechin (GC), and (+)-gallocatechin gallate (GCG).[2,3]

Catechin compounds have generated interest owing to their potential anticancer and antioxidant functions.[1] They are involved in decreasing blood cholesterol,[4] antioxidant roles,[5] inhibition of platelet aggregation,[6] reducing the incidence of cancer, and cardiovascular diseases.[7]

Conventional tea tree breeding is well established, but it is time consuming and labor intensive owing to its perennial nature and long gestation period (4–5 years).[8] Additionally, tea breeding has been slow due to lack of reliable selection criteria.[9] In particular, studies concerning the selection and breeding of tea trees containing valuable plant secondary metabolites are few. Therefore, the development of methods for the rapid selection of catechin-rich tea trees is an important strategy for breeding new cultivars. In earlier studies, catechins from various plant sources were determined employing several methods, e.g., high-performance liquid chromatography (HPLC),[10] gas chromatography (GC), or capillary electrophoresis (CE).[11,12] However, such methods are cumbersome due to the complicated procedures involved. Hence, reliable analytical methods suitable for rapid selection of tea trees rich in catechins are required. In this study we report an efficient method for the selection of catechin-rich individual tea plants among the Hadong tea population.

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Materials and methods

Plant materials

Leaves from 15-year-old tea trees were collected from January 2006 to March 2007 from specimen tea plantations located in the Hadong region, South Korea. Leaves from about 160 tea trees were randomly sampled, collected, and stored at –70°C until required. Authenticated catechin com-

pounds (EGC, EC, EGCG, and ECG), and Fast Blue B (FBB) reagents were procured from Sigma-Aldrich (USA). Extraction solvents of the highest purity were obtained from local commercial houses.

Extraction and estimation of catechins from tea trees

Fresh tea tree leaves (500 mg) were homogenized and extracted with 8 ml of distilled water on a hot water bath (Analab KSB-201) at 80ºC for 30 min. After allowing to cool at room temperature, the extracts were made up to 10 ml with distilled water and filtered (filter paper No 2, Advantec). The resulting filtrate was further extracted twice with 10 ml of ethyl acetate. Later the ethyl-acetate-extract fraction was evaporated under vacuum. The dry residue obtained was dissolved in 500 µl of ethyl acetate, filtered through a prefilter (0.2 µm, Supelco), and subjected to colorimetric and HPLC analysis.

Establishment of FBB colorimetric method for catechin determination

To establish the FBB colorimetric method, authenticated catechins (ECG, EC, EGCG, and EGC) were together treated with FBB reagent. Briefly, 1, 5, 10, 15, and 25 µg of the authenticated catechin mixtures (1:1:1:1 v/v) were reacted with FBB reagent for 5 min in a 96-well plate. Changes in the color of the reaction mixtures were determined by spotting 10 µl of supernatant on to thin-layer chromatography (TLC) plates (silica gel 60, Merck).

The FBB method was optimized with respect to catechins in tea leaf extracts and authenticated catechins by undertaking a series of trials incorporating different proportions of FBB reagent. The resulting color intensity obtained after 5 min was optimized for visual quantifications by spotting onto TLC plates (silica gel 60, Merck).

Selection of catechin-poor and -rich trees by FBB colorimetric method

FBB reagent and leaf-extracts (0.5:1, 1:1, and 2:1 v/v) were mixed and incubated for 5 min. The color intensity of the spots was visually noted by examining color on the TLC plate.

HPLC quantification of catechins

The quantitative analysis of catechins was validated by HPLC analysis. HPLC analysis of samples was conducted as described previously.[13] A filtrate sample was introduced to an HPLC system (Gilson, France) equipped with a TSKgel ODS-80Ts column (10 µm, 4.5 × 250 mm; Tosoh) and UV detector (Gilson, UV 3000). The isocratic mobile phase was a mixture of acetonitrile and 0.2% phosphoric acid in H_2O (25:75, v/v). After the injection of 20 µl of the tea extracts, the column was operated with a flow rate of 0.5 ml/min. Quantitative analysis of catechins was achieved by cochromatography of the standards and samples and by comparison of the retention times. The samples for HPLC were selected from the primary screening of tea trees through the colorimetric method.

Statistical analysis

Data are expressed as an average of at least three experiments. Each numerical value represents the mean and the standard deviation (SD).

Results and discussion

Establishment of the colorimetric method for determination of catechins from tea trees

Authenticated catechins on treatment with FBB reagent produced deep red and red–brown products (Fig. 1a). The intensity of the red complex formed by the reaction of FBB reagent with authenticated catechins increased commensurately with the catechin concentration. The estimation of catechins by the colorimetric method also correlated well with estimations based on HPLC analysis (Fig. 1b), with the correlation coefficients (r^2) for EGC, EC, EGCG, and ECG being high at 0.99, 0.99, 0.99, and 0.99, respectively.

The tea tree leaf extracts also produced red spots on treatment with FBB reagent (Fig. 2). The content of catechins increased in parallel with the depth of the color (data not shown). Also, the intensity of the red color increased commensurately with leaf extract concentrations (data not shown).

In order to determine the catechin concentration, the intensity of coloration was noted by spotting the FBB reagent on a TLC plate. However, FBB is unstable, being sensitive to the storage temperature for reasons unknown. Thus, in order to avoid this limitation, the observation of color intensities must be carried out immediately after the application of the samples, standards, and color reagents to the plates.

The optimized quantities of tea leaf extract and FBB color reagent required for application on TLC plates was assessed (Fig. 2). A ratio of 0.5:1(v/v) was found to be the optimal composition of plant extract to FBB reagent for colorimetric analysis.

A visual screening method for the selection of tea trees containing varying amounts of catechins was thus established. The tea leaf extracts on treatment with FBB reagent produced a red complex. FBB, a diazonium salt, is known to couple with carboxyl groups to form a colored complex.[14] Reynolds[15] investigated *Aloe* exudate compounds using TLC, and noted a zone staining yellow with Fast Blue B that reacts with both phenols and amines. Price and Butler[16] reported that visual estimation of tannin content using this reagent is based on the reduction by tannin and other

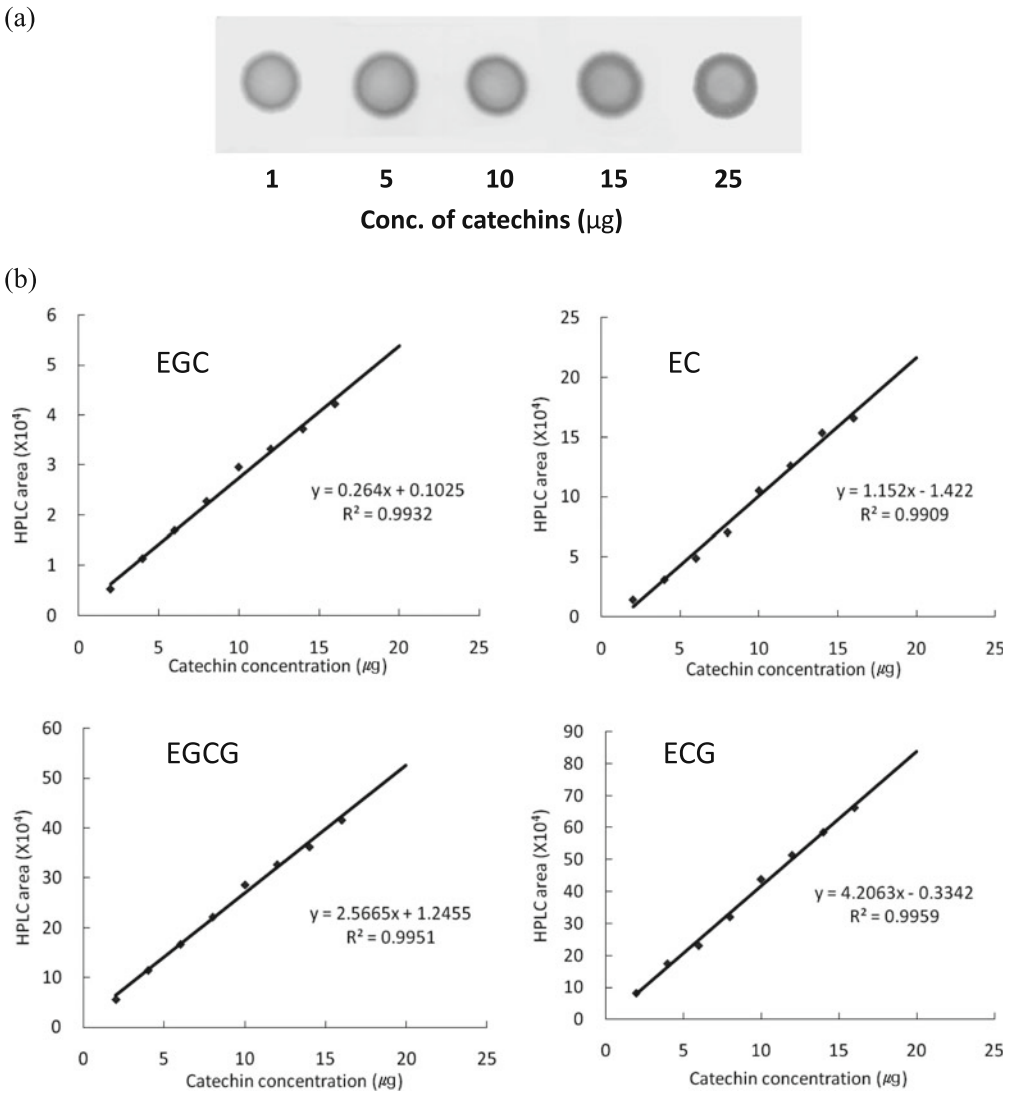

Fig. 1a,b. Quantification of catechins. **a** Color intensity with increasing concentration of four authenticated catechin mixtures treated with Fast Blue B-salt (FBB) regent on a thin-layer chromatography (TLC) plate. **b** High-performance liquid chromatography (HPLC) calibration curves for authenticated catechins

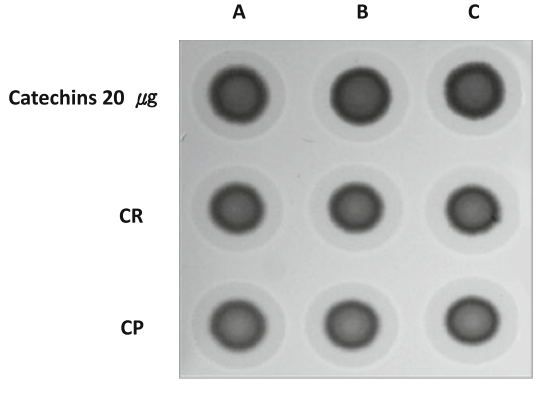

Fig. 2. Determination of optimal concentration of tea leaf extracts and FBB reagent for colorimetric estimation of catechins. *CP*, catechin-poor tea tree line (HP-138); *CR*, catechin-rich tea tree line (HR-29); *A*, FBB reagent : tea leaf extract (0.5 : 1, v/v); *B*, (1 : 1, v/v); *C*, (2 : 1, v/v)

polyphenols of ferric ions to ferrous ions, followed by the formation of a ferricyanide–ferrous ion complex. Fast Blue B reagent is known to react with various compounds, e.g., proteins, bilirubin, enzymes, cannabinoids, and phenols. In particular, the FBB reaction is a direct measure of soluble polyphenol content. Polyphenols comprise about 15%–20% of tea leaf components, whereas catechins are found to make up of 5%–10%. Polyphenols are generally divided into hydrolyzable tannins (gallic acid esters of glucose and other sugars) and condensed tannins. Tannins are a group of chemical substances found in plants, characterized by the presence of more than one phenol unit or building block per molecule. Although this colorimetric method is nonspecific, detection of catechins at lower concentrations can be measured. Thus, the FBB method is of great practical value for primary screening of tea trees based on catechin content. FBB reagent also reacts with tannin, gallic acid, and cate-

chins (data not shown) to generate red and dark red solutions. The FBB reaction with catechins was sensitive and reproducible compared to that with other polyphenols such as gallic acid and tannic acid. The visual spot analysis showed high correlation with respect to catechin concentration as determined by HPLC. Hence, this method is very simple and provides reliable selection of low- and high-catechin-containing lines. However, the exact mechanism of this reaction is yet to be elucidated.

Primary selection of catechin-rich tea tree lines by FBB colorimetric method

The tea leaf extracts from 160 specimens were treated with FBB reagent to detect catechins (Fig. 3). Based on the color intensities of the reaction products, the tea trees were divided into catechin-rich and catechin-poor tea tree plants. Among the various tea tree lines, HR-52, HR-29, HR-82, HR-123, and HR-55 were classified as catechin-rich lines and HP-19, HP-108, HP-138, HP-150, and HP-18 tea trees as catechins-poor lines.

Catechins content in selected tea tree lines

The composition of catechins among catechin-rich and catechin-poor tea trees was assessed by HPLC (Figs. 4,5). The EGC level ranged between 82.8 and 186 mg/g dry weight in catechin-rich plants, whereas the level in catechin-poor trees varied between 38 and 70.2 mg/g. The highest EGC level (186.0 mg/g dry weight) was found in catechin-rich tea tree designated HR-29, and the lowest EGC content (38.0 mg/g) was noted in HP-138 (Fig. 4). Likewise, the EC content varied from 1.06 to 43.74 mg/g in catechin-rich tea trees and from 2.66 to 11.94 mg/g in catechin-poor tea trees. In the catechin-rich tea tree line, the highest recorded EC level was 43.74 mg/g in HR-82, and among the catechin-poor group, the lowest EC level was 2.66 mg/g in HP-138. The EGCG content ranged from 0.81 to 4.23 mg/g in catechin-rich tea trees, whereas the EGCG level in catechin-poor plants ranged between 0.09 and 0.71 mg/g. The content of EGCG in catechin-rich HR-29 line was 4.23 mg/g, but only 0.09 mg/g in catechin-poor HP-18 tea trees. ECG was found to occur in the range 0.09 to 0.22 mg/g among the catechin-rich group, and 0.05 to 0.18 mg/g in the catechin-poor group. The content of ECG in catechin-rich line HR-52 was 0.22 mg/g, but only 0.05 mg/g in catechin-poor HP-19.

One of the tea tree lines (HR-55), which was selected through the colorimetric method, did not contain EC. However, most plant extracts that produced light red products showed low EC content (Fig. 4). Catechin components were usually higher in all groups of selected catechin-rich lines. However, quantitative variations of the catechin fractions (EGC, EC, EGCG, and ECG) were observed among these lines. The HR-29 tea tree line contained the highest levels of EGC and EGCG, whereas the EC content was maximal in HR-82 and the EGC content was maximal in HR-52. On the other hand, among members of the catechin-poor group, EGC and EC contents were lowest in HP-138, EGCG was lowest in HP-18, and EGC was low in HP-19 and HP-108 (Fig. 4).

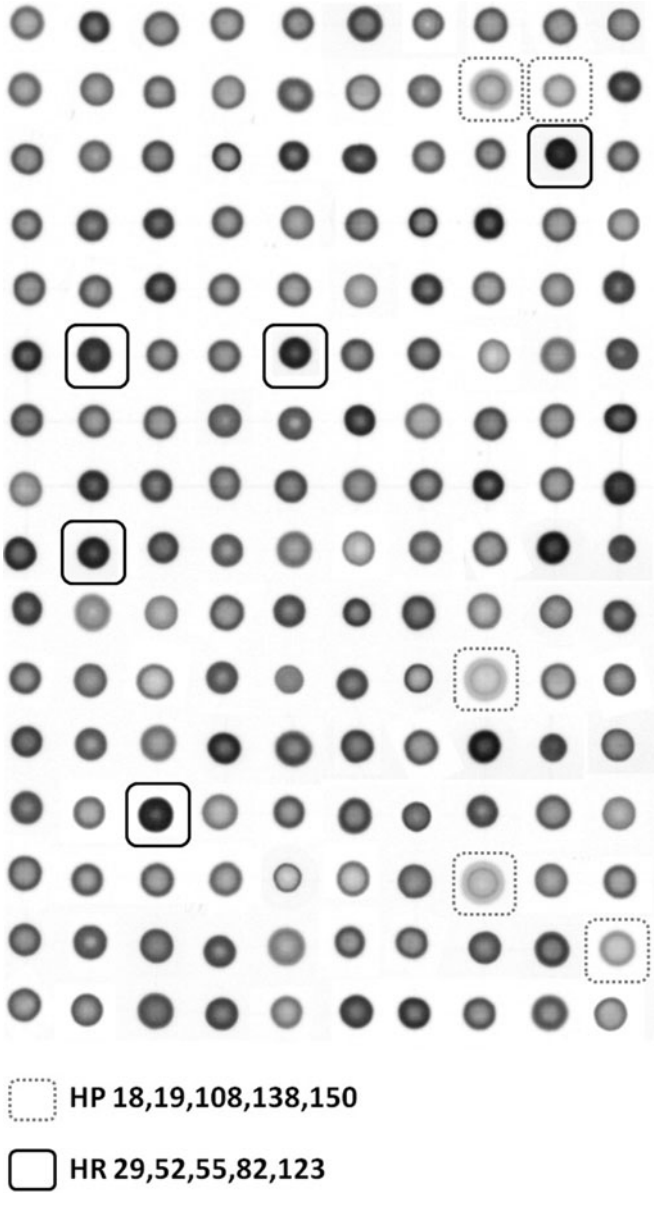

Fig. 3. Visual determination of catechins from 160 leaf extracts from Hadong tea trees with FBB reagent. *Solid square border*, intense color (HR-29, HR-52, HR-55, HR-82, and HR-123), *Dotted square border*, light color (HP-18, HP-19, HP-108, HP-138, and HP-150)

Results from both the colorimetric and HPLC methods were indicative of wide genetic variation for catechin products in tea trees of Hadong region. Among the tea tree lines selected as high and low by the colorimetric method, the catechin contents varied over a considerable range: 5-fold for EGC, 16-fold for EC, 47-fold for EGCG, and 4.4-fold for ECG. This variation may be a characteristic feature of the wild type tea tree population and geographic regions or

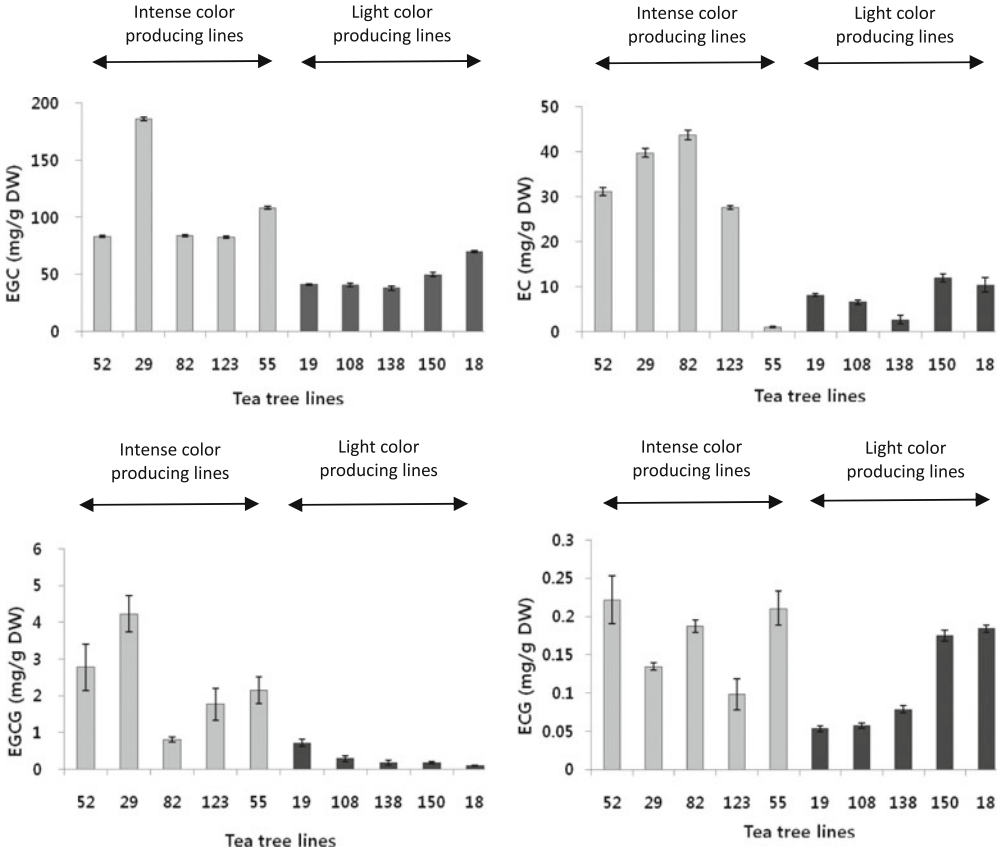

Fig. 4. Catechin content of tea trees selected by the FBB colorimetric method. Tea tree lines are randomly arranged into series of intense red and light red groups. The catechin content was analyzed by HPLC equipped with a TSKgel ODS-80Ts (10 μm, 4.5 × 250 mm, Tosoh) column and a UV detector. *EGC*, (−)-epigallocatechin; *EC*, (−)-epicatechin; *EGCG*, (−)-epigallocatechin gallate; *ECG*, (−)-epicatechin gallate

habitats of Hadong. The wild tea tree population in Hadong region has not been bred by breeders. The Hadong region tea tree population has been restricted to the Jiri Mountains for thousands of years; this situation has placed it in geographic and genetic isolation. As Hadong region tea trees have been propagated through seeding, they show a high degree of genetic variability. Gershenzon and Groteau[17] have reported that composition and concentration of secondary metabolites vary among plants due to their cultivation in varied geographic regions.

The catechin contents of the selected as high and low tea tree lines were determined during different seasons, e.g., spring and winter (Fig. 6). The catechin contents of tea tree leaves collected during different seasons showed wide variation. The catechin contents of trees collected in November 2006 were lower than those of March 2007, except for EGC. Despite these seasonal variations, the FBB colorimetric method was able to clearly establish catechin-rich and -poor tea tree lines beyond doubt.

The catechin composition patterns in the primary selected as high and low lines were influenced by the season of sample collection. The amount of catechins tends to vary through the growing season. The catechin content of tea leaves collected in spring was much higher than that from plants collected in winter. In a previous study by our group,[13,18] the caffeine content in the Hadong tea population was also influenced by the sampling time. Perhaps this change of catechin content in tea trees is brought about by various factors, such as biosynthetic, environmental, and genetic factors. De Freitas and Glories[19] have reported that polyphenols in general are actively metabolized throughout the growing season (March to September). There are other factors, such as climate, which could be responsible for the differences noted.

Conclusions

This study elucidated an efficient selection procedure to obtain catechin-rich tea trees by an FBB colorimetric method. The method was successfully adopted to classify the Hadong region tea trees into catechin-rich and catechin-poor trees. Catechin-rich tea trees had increased levels compared to catechin-poor trees of 5-fold for EGC, 16-fold for EC, 47-fold for EGCG, and 4.4-fold for ECG. Thus, the FBB colorimetric method appears to be practical for the primary selection of tea tree lines with varying catechin contents. The results have potential applications for selective breeding of tea trees containing high amounts of catechins.

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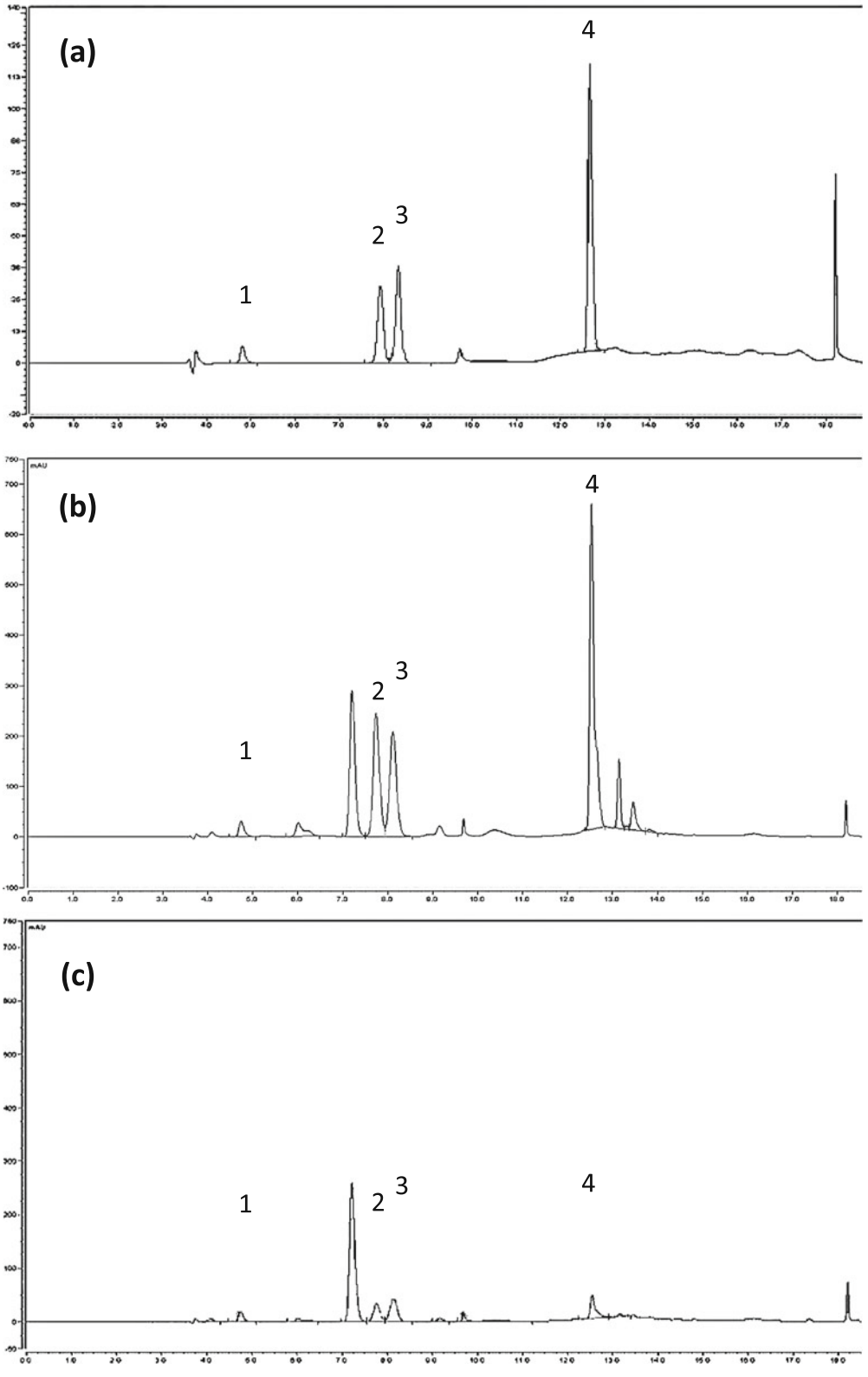

Fig. 5a–c. HPLC chromatograms of tea leaf extracts after selection by the FBB colorimetric method. **a** Authenticated catechins: *1*, EGC; *2*, EC; *3*, EGCG; and *4*, ECG; **b** catechin-rich line (HR-29), **c** catechin-poor line (HP-138)

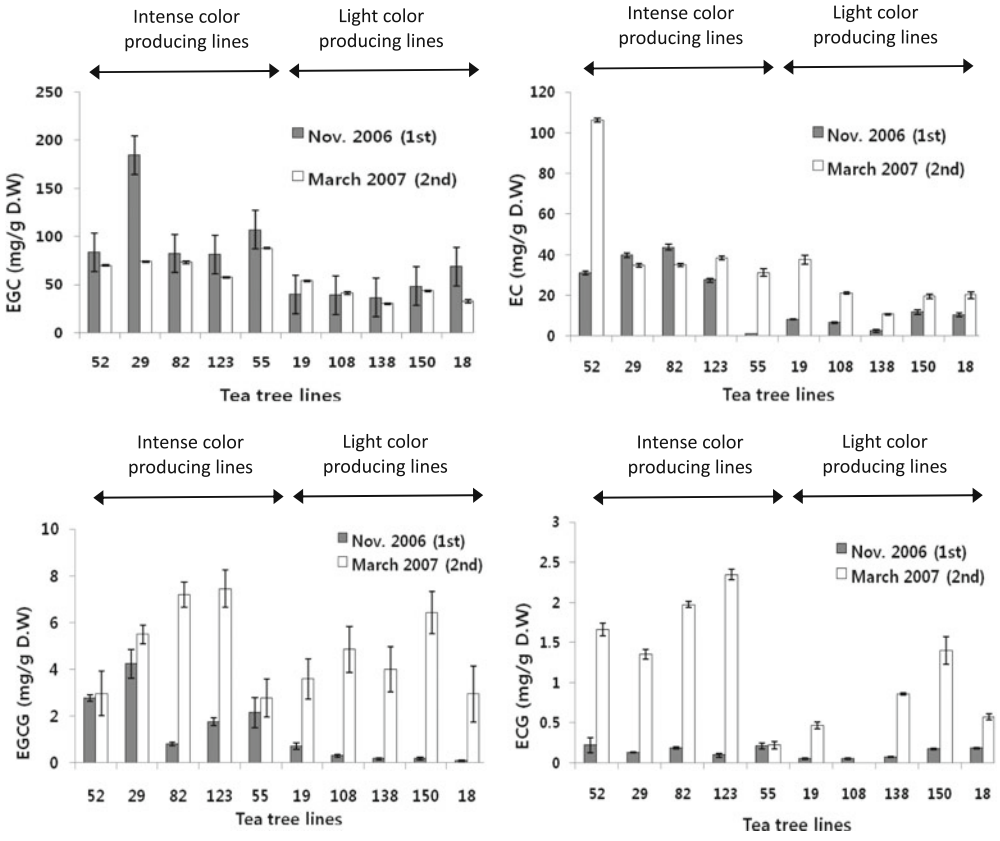

Fig. 6. Catechin content for different sampling times

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