Running title: Analysis of coumarin and its precursor in green tea

Title: Analysis of coumarin and its glycosidically bound precursor in Japanese green tea having sweet-herbaceous odor

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Abstract

Coumarin is a natural product well-known for its pleasant sweet-herbaceous and cherry flower-like odor. Despite coumarin is widely found in the plant kingdom, its occurrence in tea leaves is very poorly characterized. In this work, the cultivars, "Shizu-7132", "Koushun" and "Tsuyuhikari" were found to have sweet-herbaceous odor from the 11 Japanese green teas by the sensory descriptive analysis. Application of the stable isotope dilution assay for the quantification of coumarin revealed that the levels of coumarin in these Japanese green tea products with sweet-herbaceous and cherry flower-like odor ranged from 0.26 to 0.88 μg/g of green tea product, whereas the concentrations were generally below 0.2 μg/g of common green tea products. The "Shizu-7132" was found as naturally coumarin-rich green tea. The natural level of

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coumarin in fresh tea leaves of Shizu-7132 was highly abundant with concentrations amounting to 4.13 μg/g. In contrast to the leaf part of the fresh leaves, the stem part contained much less coumarin. During the manufacturing process of Shizu-7132, the short steaming time and low drying temperature were favorable for the accumulation of coumarin in the final product. For the hydrolysis of glycosidically bound coumarin precursors, in fresh tea leaves, most coumarin occurred in its free form. In addition, the tea leaves also contained small amounts of the bound coumarin precursor such as primeveroside.

Keywords: Coumarin; Green tea; Sweet-herbaceous; Glycosides; Precursor

Introduction

Coumarin (1, 2-benzopyrone) is a natural product having a sweet-herbaceous and cherry flower-like odor. It was reported from many plants, including sweet clover (*Melilotus alba*) (**Akeson, Gorz, & Haskins, 1963**), tonka bean (*Coumarouna odorata*) (**Haskins, & Gorz, 1963**), lavender (*Lavandula officinalis*) (**Brown, 1962**), and cinnamon (*Cinnamonum verum*) (**Miller, Poole, & Pawloski, 1996**). It has also been detected in Pouching and Longjing tea (**Yamanishi, Kosuge, Tokitomo, & Maeda, 1980; Kawakami, & Yamanishi, 1983**), although the teas contain minute quantity of coumarin. Besides occurring naturally in these plants, coumarin has been widely used as a flavoring compound because of its sweet and aromatic odor. Cinnamon tea is a most popular flavored tea with a blend of the tea deliciously flavored with cinnamon.

Coumarin was reported as a character impact compound for the sweet odor quality of Japanese green tea and Chinese green tea (**Kumazawa, & Masuda, 2002**). There are a few Japanese green tea products with coumarin-like and a natural sweet-herbaceous fragrance in the commercial markets. However, no information is available regarding the levels of coumarin in these Japanese green teas and changes in content of coumarin during the tea manufacturing process.

Gas chromatography-mass spectrometry (GC-MS) or liquid

chromatography-mass spectrometry (LC-MS) methods have been used for the determination of coumarin in plants and foods (**Christakopoulos, Feldhusen, Norin, Palmqvist, & Wahlberg, 1992; Rychlik, 2008**). It is generally accepted that quantitation of plant or food samples is more accurate if an internal standard is used, which has very similar chemical and physical properties and behaves nearly identically throughout the whole analytical procedure. Therefore, stable isotopologues of coumarin are considered the best internal standard in the quantitation of coumarin in plant or food samples (**Rychlik, 2008**).

In this study, we developed an accurate and simple method for the determination of coumarin in tea leaves by GC-MS using [5, 6, 7, 8-$^{2}H_4$]-coumarin as internal standard. To select the natural coumarin-rich green teas, we investigated the natural levels of coumarin in several Japanese green tea leaves with sweet- herbaceous odor and the quantitative changes in coumarin during the tea manufacturing process. Furthermore, because coumarin is known to occur in plants both in the free form and as glycosidically bound precursors (**Dewich, 2002**), we also attempted to confirm the presence of glycosidic precursors of coumarin in tea leaves using the acidic and enzymatic hydrolysis assays.

Materials and methods

Chemicals

Unlabeled coumarin was purchased from Wako Pure Chemical Industries, Ltd., Japan. [5, 6, 7, 8-$^{2}H_4$]-coumarin (deuterium atom 98%) was purchased from C/D/N Isotopes INC., Canada. (*E*)-2-coumaric acid glucoside was a generous gift from Dr. Shimizu, B. of Kyoto University, Japan. β-glucosidase from almonds (36.8 units/mg) was purchased from Oriental Yeast Co., Ltd., Japan and β-primeverosidase was a gift from Dr. Mori, Amano Enzyme Inc., Japan. All other chemicals were of analytical grade.

Tea samples

The 11 cultivars of Japanese green tea leaves, including “Shizu-7132”, “Koushun”, “Kanayamidori”, “Sayamamidori”, “Yabukita”, “Sayamakaori”, “Asatsuyu”,

"Tsuyuhikari", "Inzatsu-131", "Fujikaori", and "Ohiwase", were plucked in Shizuoka Tea Experimental Station (Fuji, Shizuoka, Japan) in May, 2005 and 2007. The manufacture of Japanese green tea was performed through the traditional method: a) plucking, b) steaming, c) rolling, and d) drying as shown in **Fig. 1**.

To investigate the effect of processing on the content of coumarin in tea, sampling from different steaming time (30 s, 60 s, 90 s, and 120 s) and drying temperature (50 °C, 60 °C, 70 °C, and 80 °C) were carried out.

Sensory evaluation

Tea leaves equivalent to 3 g of dry tea product were infused with 150 ml freshly boiled water for 3 min. Sweet-herbaceous descriptive analyses were carried out by 5 professional tea tasters from Shizuoka Tea Experimental Station.

Determination of coumarin in teas by GC-MS using stable isotope assay

Two gramme of tea leaves powder ground by liquid nitrogen were added 2.4 μg of [5, 6, 7, 8-$^{2}H_4$]-coumarin and 5 ml of an azeotropic mixture of pentane-dichloromethane (2:1 v/v). Our preliminary experiment showed that the amount of coumarin had no significant difference ($p > 0.05$) among 3 h-, 6 h- and 12 h-extraction of the tea sample, so the sample was stirred only for 3 h at 20 °C under the dark conditions, and then filtered through a short plug of anhydrous sodium sulfate. One μl of the filtrate was obtained and subjected to GC-MS analyses.

Qualitative and quantitative analyses of coumarin in tea samples were performed using a GC-MS QP5050 (Shimadzu), which was controlled by a Class-5000 work station. The GC was equipped with a capillary TC-5 column (GL Sciences Inc., Japan), 30 m × 0.25 mm I.D., and 0.25 μm film thickness. Helium was used as a carrier gas with a flow of 50 ml/min. The injector temperature was 230°C. The GC oven was maintained at 60 °C for 3 min. The temperature of the oven was programmed at 15 °C/min to 110 °C and then at 40 °C/min to 290 °C, and kept at this temperature for 3 min.

The mass spectrometer was operated by the full scan mode (mass range *m/z*

60-200 for qualitative analysis of coumarin) or by the selected ion monitoring (SIM) mode for quantitative analysis. In the latter case, the mass spectrometer was focused at *m/z* 146, 118, and 150, 122, the dominating fragments ($M^{\bullet+}$ and $M\text{-}CO^{\bullet+}$) for coumarin and [5, 6, 7, 8-$^2H_4$]-coumarin, respectively. Quantification was based on the ratio of peak areas of *m/z* 146, 118 to those of *m/z* 150, 122.

Calibration curve for the coumarin

A standard curve for coumarin was prepared by adding to 5 ml of solvent (pentane- dichloromethane 2:1, v/v) various amounts of unlabeled coumarin (0.026-13.5 μg/ml) to a constant amount (0.48 μg/ml) of [5, 6, 7, 8-$^2H_4$]-coumarin. The preparation was carried out according to the tea sample preparation.

Acidic and enzymatic hydrolysis assays for glycosidically bound precursors of coumarin in tea leaves

Tea samples equivalent to 3 g of dry weight were homogenized overnight at 4 °C in a mixture of methanol/saturated $CaCl_2$ (80:20, v/v, 25 ml) containing 0.75 mg of phenyl-β-D-glucopyranoside as internal standard. The homogenates was centrifuged at 15, 000 g (20 min). The resulting supernatant was concentrated to dryness and treated as the crude extract (CE).

Each of one-fifth of CE was used for the each experiment as follows, 1) stirred in hydrochloric acid (2.5 mol/L, 3 ml) at 80 °C for 90 min; 2) incubated in 50 mM pH 6.0 citric acid buffer as a control of the acidic reaction; 3) incubated in 3 ml of citric acid buffer (50 mM, pH 6.0) with 2.5 mg of β-glucosidase at 37 °C for 20 h; 4) incubated in 3 ml of citric acid buffer (50 mM, pH 6.0) with 80 mg of β-primeverosidase at 37 °C for 20 h; 5) without enzyme at 37 °C for 20 h as a control of the enzymatic reactions. Subsequently, each of the reaction solutions was cooled to room temperature, and then added 1.44 μg of [5, 6, 7, 8-$^2H_4$]-coumarin as the internal standard. The resulting mixtures were filtered through 0.45 μm filter and analyzed by LC-MS.

For a simple and fast detection of liberation of bound coumarin precursors, stable isotope dilution assays based on LC-MS was used for the quantification of

coumarin after treatment with hydrolysis (**Rychlik, 2008**). Because GC-MS requires the further extraction with organic solvents for separating the odorant from nonvolatile matrix compounds. LC-MS analysis of coumarin after treatment with hydrolysis was performed in an LCMS-2010 A system (Shimadzu Cooperation, Tokyo, Japan) equipped with a 2.0 mm× 150 mm i.d., 5 μm, UG120 C-18 reversed-phase column (Shiseido Co. Ltd., Japan). A total of 10 μl of the sample solutions were chromatographed using gradient elution with aqueous formic acid (0.1%, v/v) as solvent A and acetonitrile as solvent B, at a flow rate of 0.2 ml/min at 40 °C. Elution was started with isocratic conditions of 12% of solvent B and 88% of solvent A for 5 min. The former was increased to 33% at 18 min and linearly increased to 100% at 23 min. Then, 100% of solvent B was maintained for 10 min and subsequently brought back within 1 min to 12% of the solvent and held for another 15 min to allow for column equilibration. UV-vis spectra were recorded between 200 and 600 nm for each chromatographic peak. For [5, 6, 7, 8-$^{2}H_4$]-coumarin, the *m/z* 151 and, for unlabeled coumarin, the *m/z* 147 were chosen in the SIM positive mode. Electrospray operating conditions were optimized in each analysis from a standard one: dry gas 1.5 L/min, capillary voltage 1.5 kV, dry temperature 250 ºC.

Identification of 2-coumaric acid glucoside in tea leaves

There is evidence that 2-coumaric acid glucosides were found as glycosidically bound precursor of coumarin in some plants (**Fig. 2, Dewich, 2002**). To investigate its presence in tea leaves, besides the crude extracts of leaf and stem of tea leaves shown in above, the glycosidic fractions from tea leaves were also prepared through the following isolations: a) removing polyphenols by Polyclar AT, b) precipitating protein by methanol, c) removing sugars, volatile compound using Amberlite XAD-2 eluted by water and pentane / dichloromethane respectively, as describe by **Wang et al. (2000)**. The glycoside extracts and crude extracts of tea leaves were analyzed by LC-MS. For LC-MS analysis of 2-coumaric acid β-D-glucopyranoside in tea leaves, a similar chromatographic condition was described as above, but for 2-coumaric acid

β-D-glucopyranoside, the *m/z* 325 and *m/z* 327/ *m/z* 165 were selected in the SIM negative and positive modes respectively. The MS range of *m/z* 100-600 was operated in full scan negative and positive modes.

Statistical analysis

All experiments were performed in triplicate. One-way ANOVA was used to estimate overall significance followed by post hoc Turkey's tests corrected for multiple comparisons (**Miller, 1981**). Data are presented as mean ± S.D. A probability level of 5% ($p < 0.05$) was considered significant.

Results and discussion

Mass spectrometric profile of coumarin, calibration curves for the coumarin from GC-MS analyses, and extraction of coumarin in tea leaves

As discussed by **Christakopoulos et al. (1992)**, the GC-MS-SIM using stable isotope as internal standard method is accurate and sensitive for determination of natural concentrations of coumarin in plants. Because the method compensates for errors caused by the gas chromatograph (injection, column absorption), mass spectrometer (instability of the instrument), and incomplete recovery of the extraction procedure. **Fig. 3** shows the mass spectra of the standards and tea sample. It excludes the possibility of naturally occurring of the dominating fragments (*m/z* 150 and 122) of [5, 6, 7, 8-$^2H_4$]-coumarin from tea samples. This suggests that quantification of coumarin based on the peak areas of *m/z* 146, 118 (unlabeled coumarin) to those of *m/z* 150, 122 (D-labeled coumarin) is accurate.

The standard curve displays a good linearity ranging from 0.026 to 13.5 μg/ml in the extracts (**Fig. 4**). The detection limit of the method is 0.065 μg of coumarin/g of tea leaves. The sample preparation previously used for GC-MS analysis requires several steps for separating the odorant from nonvolatile compounds (**Christakopoulos, Feldhusen, Norin, Palmqvist, & Wahlberg, 1992**). The extraction procedure presented here is very straightforward. After the samples were extracted in pentane / dichloromethane containing definite amounts of [5, 6, 7, 8-$^2H_4$]-coumarin, the extracts

only had to be passed through a short plug of anhydrous sodium sulfate. To evaluate whether the extraction procedure was sufficient for quantifying total coumarin in tea, the recovery was evaluated by adding 6 μg or 12 μg of coumarin to 2 g (fresh weight) tea sample (steaming in 2005) and was found to be 103% and 96% respectively. Intra-assay precisions were determined by analyzing coumarin in tea samples (**Fig. 5 and Fig. 6**) and revealed coefficient of variations of below 9% for total coumarin (n=3).

Natrual levels of coumarin in Japanese teas having sweet- herbaceous odor and changes in coumarin content during the manufacturing process

The cultivars, "Shizu-7132", "Koushun", and "Tsuyuhikari" were found to have sweet-herbaceous odor among the 11 cultivars of Japanese green tea by the sensory evaluation. The levels of coumarin in these Japanese green tea products having sweet-herbaceous odor varied between 0.26 and 0.88 μg/g (**Fig. 5**). In contrast to this, the cultivar "Yabukita" without sweet-herbaceous odor contained less coumarin (below 0.1 μg/g) (**Fig. 5**). In cinnamon-flavored tea including cassia as an ingredient, the concentration of coumarin varied between 0.74-0.94 μg/g (**Rychlik, 2008**). To our knowledge, the concentrations of coumarin in green tea products were generally below 0.2 μg/g in the previous reports (**Yamanishi, Kosuge, Tokitomo, & Maeda, 1980; Kawakami, & Yamanishi, 1983**), whereas in the tea product of "Shizu-7132", coumarin was highly abundant with concentrations amounting to 0.88 μg/g, indicating that "Shizu-7132" is a naturally coumarin-rich Japanese green tea.

To further obtain an insight as to the origin of coumarin-rich in "Shizu-7132", the changes in content of coumarin during the tea manufacturing process were investigated. There were similar characteristics between the samplings in May, 2005 and 2007 in the changes of coumarin during the manufacturing process. As shown in **Fig. 6A**, in the sampling in May, 2007, the fresh leaves contained 4.13 μg/g coumarin. In contrast to the leaf part of the fresh leaves, the stem part contained less coumarin. The content of coumarin increased only in the stem part after the steaming process. In the leaf part, there was no significant increase in content of coumarin. This may imply that coumarin

mostly occurred in its free form in the leaf part of tea leaves, and only small amounts of the bound precursor was found in the stem part. During the rolling process, the leaves were mechanically destroyed, enabling the enzymes such as glycosidases to become more active and to have more chance of interacting with the substrates, which may accelerate the production of coumarin from glycosidically precursors. Consequently, the content of coumarin showed some increase during the prophases of rolling process (R1-R3). With the import of hot-air and the elevation of temperature during the anaphases of rolling process (R4-R5), coumarin decreased gradually. Particularly after drying process, coumarin reduced rapidly. **Figs. 6B** and **6C** reveal that the coumarin content was steaming time-dependently and drying temperature-dependently reduced, suggesting that the comparatively short-steaming time and low-drying temperature had the positive effect on the accumulation of coumarin in the final product of tea.

Identification of glycosylated precursor of coumarin in tea leaves and liberation of coumarin from glycosides by enzymatic or acidic hydrolysis

Although there is evidence that the plants actually contain the glucosides of 2-coumaric acid, and coumarin is only liberated as a result of enzymatic hydrolysis and lactonization through damage to the plant tissues during harvesting and processing (**Fig. 2, Dewich, 2002**), 2-coumaric acid glucoside was not detected in tea leaves in the present study.

It is generally accepted that some tea aroma compounds are mainly present as glycosides in fresh tea leaves and are released by endogenous enzymes (β-glucosidase and β-primeverosidase) during the tea manufacturing process (**Guo, et al., 1993, 1994; Wang, et al., 2000, 2001a, 2001b**). To confirm the presence of coumarinyl glycoside in tea leaves, we analyzed the releasing ratio of coumarin from glycoside by the acidic hydrolysis and enzymatic (β-glucosidase and β-primeverosidase) hydrolysis. The LC-MS using stable isotope as internal standard method was applied to the analysis of coumarin in the hydrolysis reactions (**Fig. 7A**). For both the leaf part and stem part of fresh tea leaves, β-glucosidase treatment had no significantly effect on liberating the

precursors (**Fig. 7B**). In contrast to this, the coumarin content increased significantly (p<0.01) after β-primeverosidase treatment (**Fig. 7B**), suggesting that coumarinyl glycoside in tea leaves may be primeverosides. As our preliminary studies showed that β-primeverosidase activity was detected in the crude enzymes obtained from the tea samples even after the processing, we are now chemically preparing 4-*O*-coumaroyl β-primeveroside to identify the glycosidic precursor of coumarin in tea samples. In particular, in the stem part of fresh tea leaves, in contrast to the free coumarin, the high percentage of bound coumarin was released (**Fig. 7B**), which is in good accordance with most bound coumarin occurred in the stem part of tea leaves as described above. For the acidic hydrolysis treatment, however, the coumarin contents in both the leaf and stem part of tea leaves were reduced significantly (p<0.01) (**Fig. 7B**).

Conclusion

In the present study, an accurate, simple and fast method for qualitative and quantitative determination of endogenous coumarin in tea leaves has been developed. The cultivar of Japanese green tea with sweet-herbaceous odor, “Shizu-7132” was found as the naturally coumarin-rich green tea. The change in the content of coumarin during the tea manufacturing process was clarified, which would provide valuable information for production of coumarin-rich green tea. Although 2-coumaric acid glucoside was not detected in tea leaves, the results presented here indicate that coumarin mostly occurred in its free form in tea leaves, and the tea leaves also contained small amounts of the bound coumarin precursor, which may be primeverosides. To our knowledge, this may be the first description of the coumarin-rich green tea and coumarinyl glycoside in tea leaves. It remains to be determined for the role of coumarin in the total flavor of the coumarin-rich green tea. More scientific and field works are proposed to be done on the promotion of naturally coumarin-rich green teas as well-accepted green tea with particular flavor.

Abbreviations

CE, Crude extract; D, Drying; F, Plucking; GC-MS, Gas chromatography-mass

spectrometry; I. S., Internal standard; LC-MS, Liquid chromatography-mass spectrometry; R1, Primary rolling 1; R2, Primary rolling 2; R3, Rolling; R4, Medium rolling; R5, Refined rolling; S, Steaming.

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Legends to figures:

Figure 1 Sampling during the manufacturing process of Japanese green tea.

The rolling processes (R1-R5) were preformed through the four types of rolling machines using different rolling strength and hot air.

Figure 2 Proposed pathway of coumarin formation in some plants (**Dewich, 2002**).

Figure 3 Mass fragmentogram of coumarin standards and tea sample in GC-MS analysis.

Figure 4 Standard curve obtained by analysis of various amounts of unlabeled coumarin and a constant quantity of D-labeled coumarin.

Figure 5 Natural levels of coumarin in Japanese green tea products having sweet-herbaceous odor.

Data are expressed as mean ± S. D. (n=3). The cultivar "Yabukita" without sweet-herbaceous odor was used a control.

Figure 6 Changes in coumarin content during the manufacturing process (A), and effect of steaming time (B) and drying temperature (C) on the content of coumarin in the tea leaves.

Figure 7 LC-MS chromatogram of enzymatic or acidic hydrolysis of coumarinyl glycoside in tea leaves (A) and the releasing ratio of coumarin from glycoside (B)

(A) Sample: the stem part of fresh tea leaves of Shizu-7132 in 2007. (B) Data are expressed as mean ± S. D.(n=3), shown by percentage of the treatment without hydrolysis. ** $p < 0.01$ when compared with that of the treatment without hydrolysis.

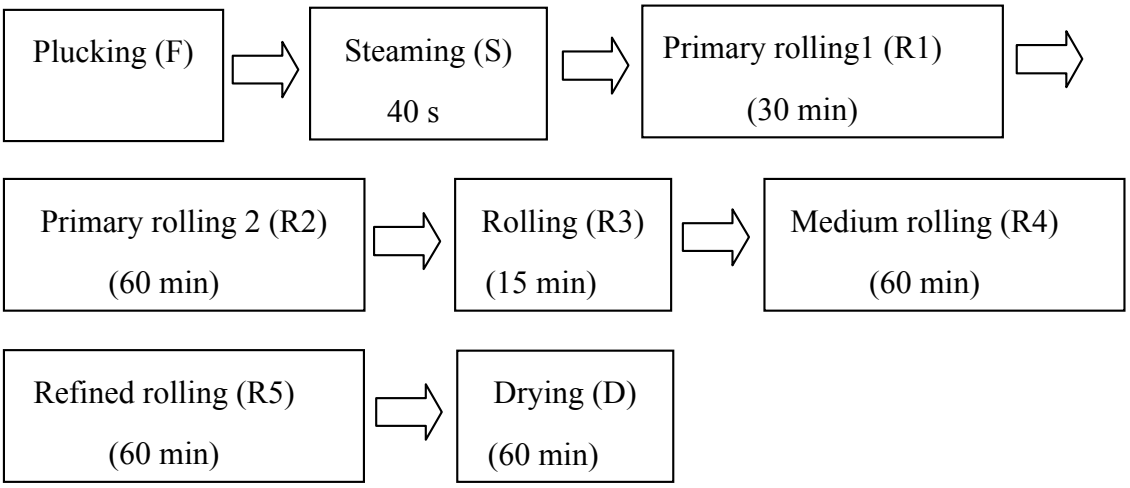
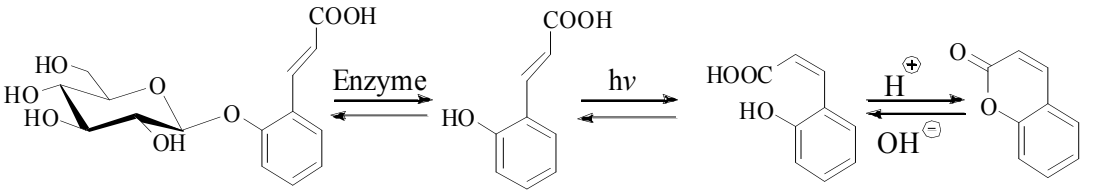


Fig. 1, **Ziyin Yang**



2-glucosyloxycinnamic acid Trans-*o*-Hydroxycinnamic acid Coumarin

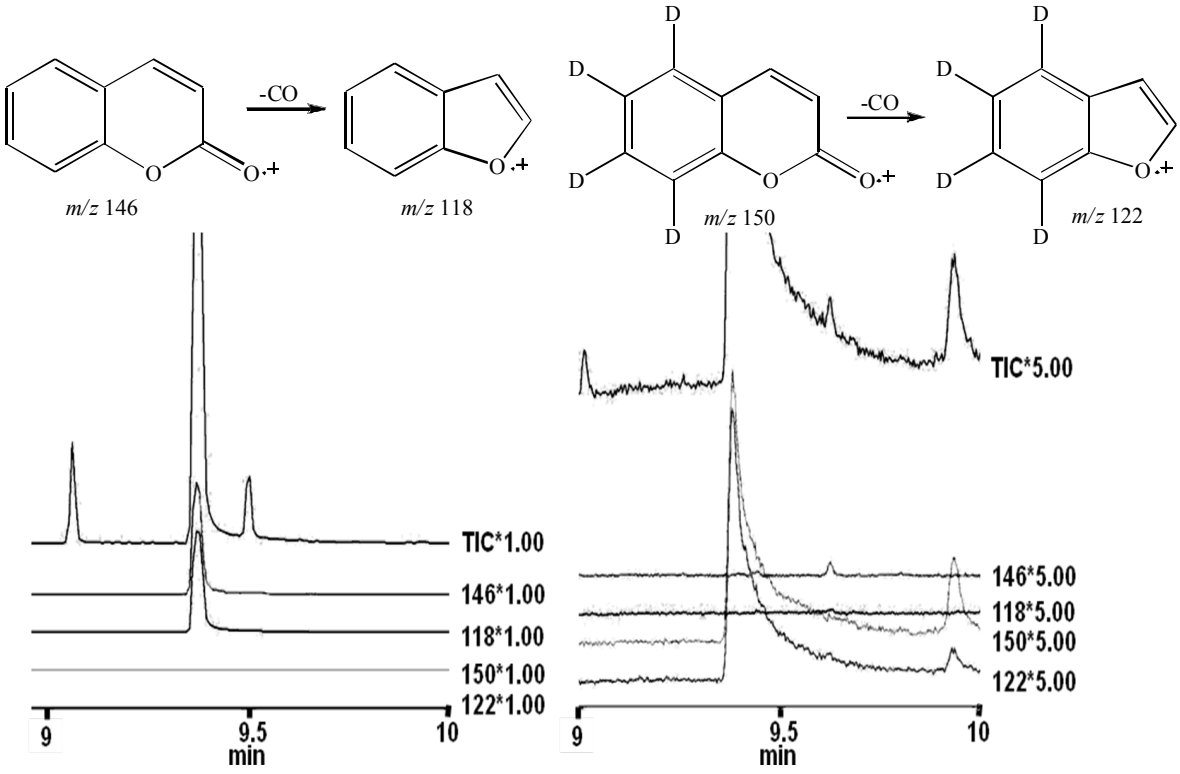
Fig. 2, **Ziyin Yang**

Unlabeled coumarin

No peaks of *m/z* 150 and 122

Coumarin-D4 (Internal standard)

No peaks of *m/z* 146 and 118



Fresh tea leaves in 2007

(Without I.S. coumarin-D4)

No peaks of *m/z* 150 and 122

Fresh tea leaves in 2007

(With I.S. coumarin-D4)

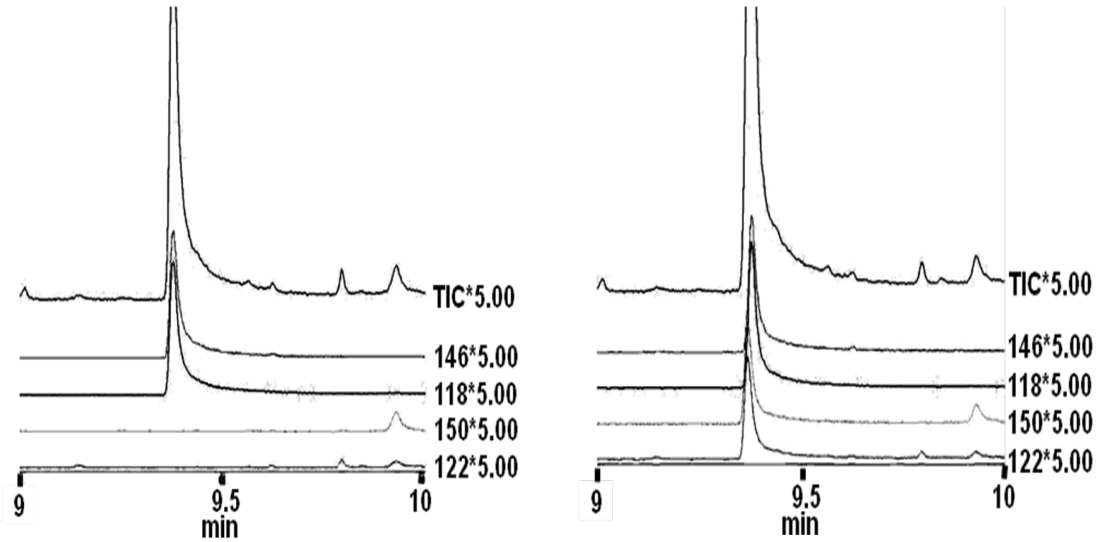
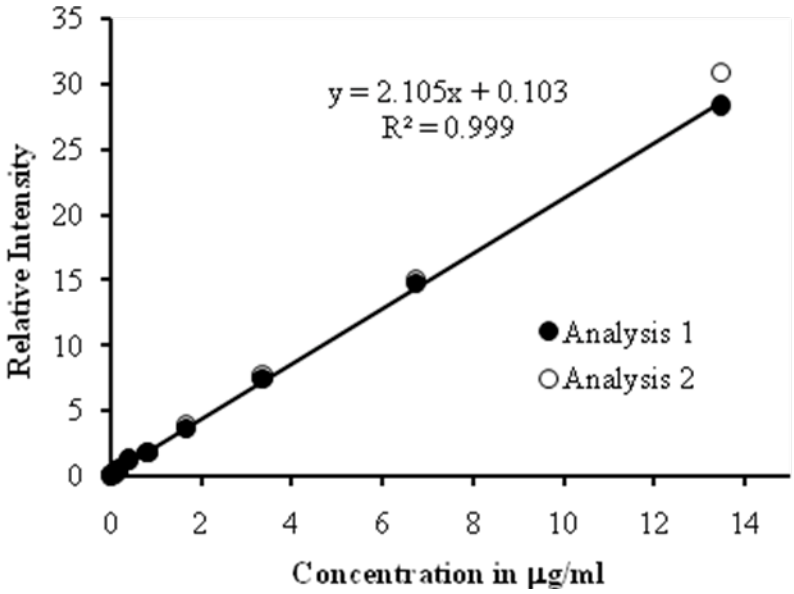


Fig. 3, **Ziyin Yang**



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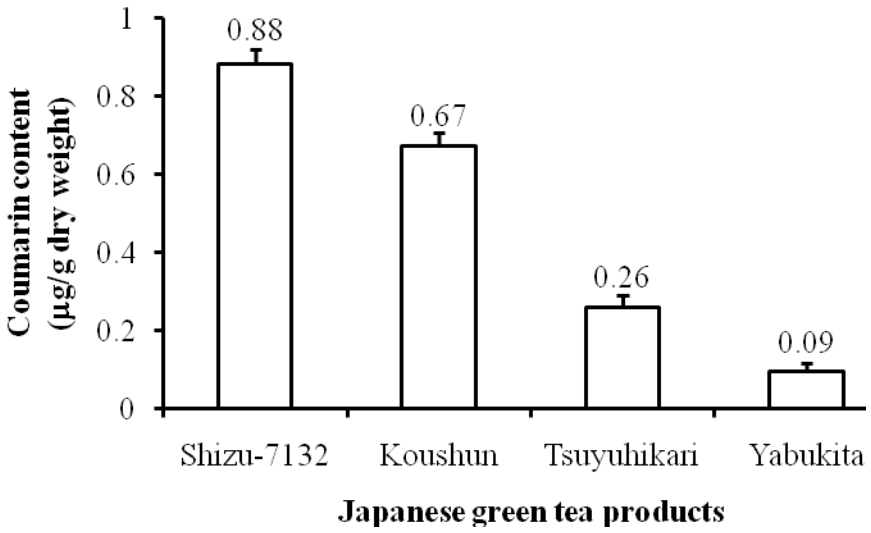

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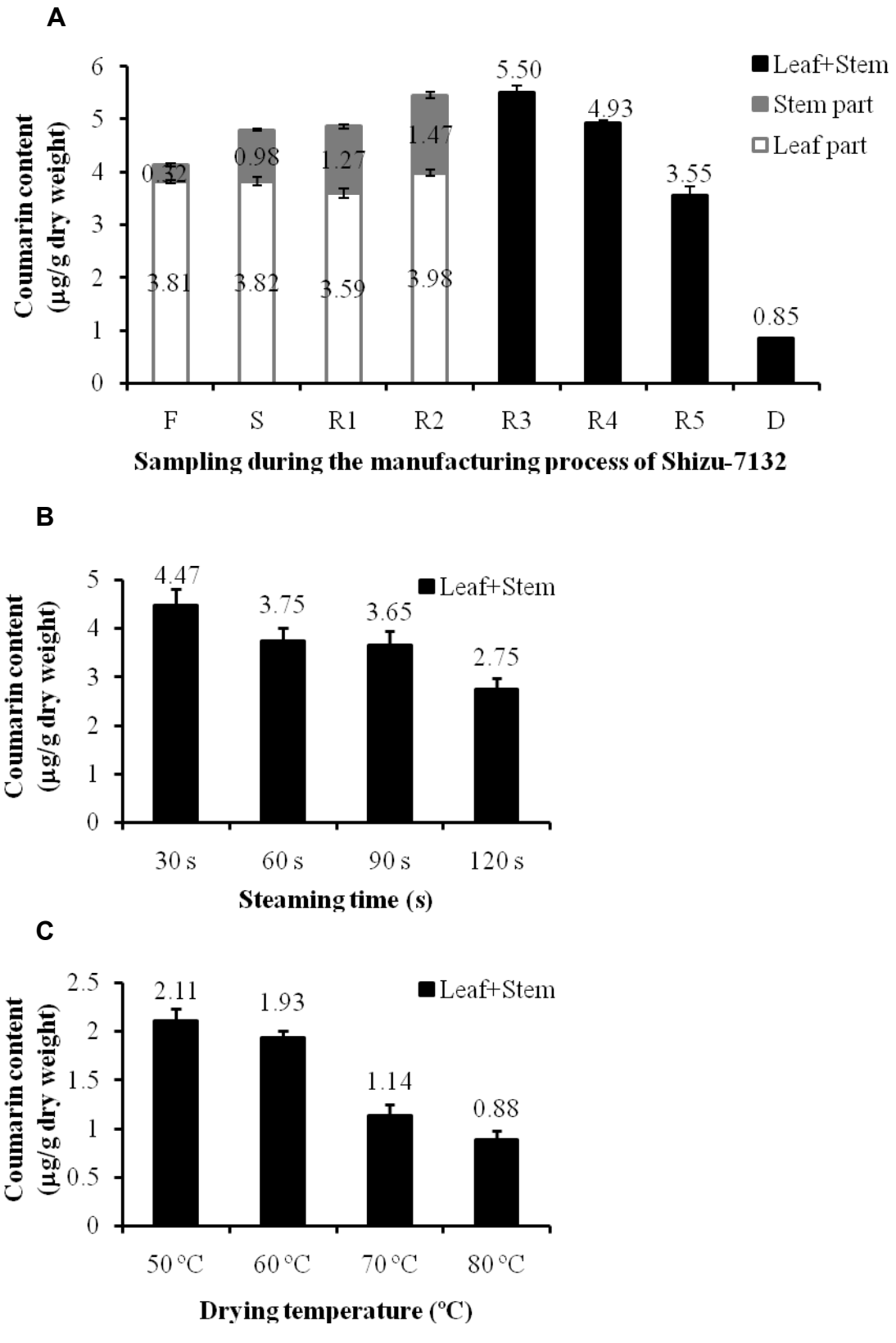

Fig. 6, **Ziyin Yang**

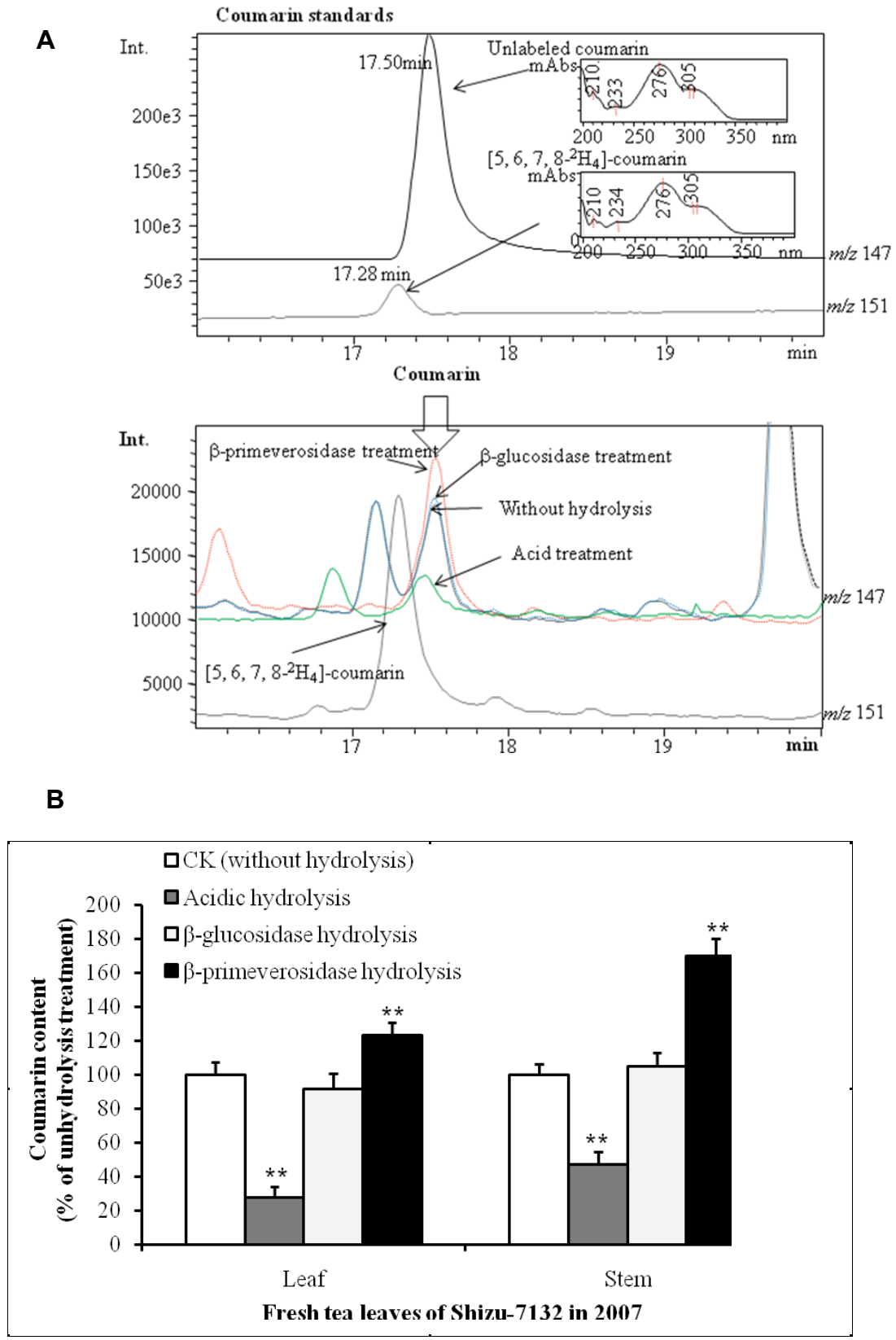

Fig. 7, **Ziyin Yang**