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Research Article

An Improved Method of Theabrownins Extraction and Detection in Six Major Types of Tea (*Camellia sinensis*)

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Tea pigments consisting of theabrownins (TBs), theaflavins (TFs), and thearubigins (TRs) affect the color and taste of tea. TBs include a variety of water-soluble compounds, but do not dissolve in n-butanol and ethyl acetate. Previously, the traditional method of TB extraction only mixed tea with n-butanol, and TBs were retained in the water phase. However, without ethyl acetate extraction, TFs and TRs remained in the water phase and affected the detection of TB content. Although an improved method had been devised by adding an ethyl acetate extraction step between tea production and n-butanol extraction, the proportional equation for calculating TB content (%) was not yet developed. In this study, we compared the absorbance at 380 nm (A_{380}) of TB solutions from six major types of tea (green, yellow, oolong, white, black, and dark teas) extracted by improved and traditional methods from the same tea samples. Significantly lower A_{380} values were obtained from TB solutions via the improved method compared to the traditional method for six major types of tea, and the highest and lowest slops in TB concentrations from A_{380} analyses were from dark tea and green tea, respectively. Moreover, newly developed equations for TB content in those six tea types extracted by the improved methods were also established.

1. Introduction

Tea is either prepared by infusion or decoction of *Camellia sinensis* dried leaves, which are classified into six major types (green, oolong (or cyan), black, white, yellow, and dark) based on the manufacturing process. Both black and oolong teas are two kinds of aerated tea leaves. After plucking, these fresh leaves are processed by withering, rolling, aeration, and then inactivation by drying (Figure 1(a)). During the aeration process, polyphenols are oxidized by endogenous enzymes such as polyphenol oxidase and peroxidase, thus producing many oxidative compounds. Polyphenol oxidation results in the pigment's dynamic transition into tea pigments that include TFs, TRs, and TBs [1, 2]. The relationship among TFs, TRs, and TBs is shown in Supplementary Figure 1. The transition induces color changes in tea leaves ranging from green to red brown [3]. All the pigments affect the color and flavor of liquid tea [3–7]. TBs give tea leaves a brown color and can be formed by TFs or TRs oxidation or polyphenol aggregation with other sugars and acidic compounds [8, 9]. The leaves of oolong tea are green with red edges (Figure 1(b)III) due to the formation and accumulation of oxidized polyphenols from leaf margins during the aeration process. As the aeration period proceeds, the surfaces of all tea leaves become reddish brown (Figure 1(b)I) and black tea leaves are produced. Therefore,

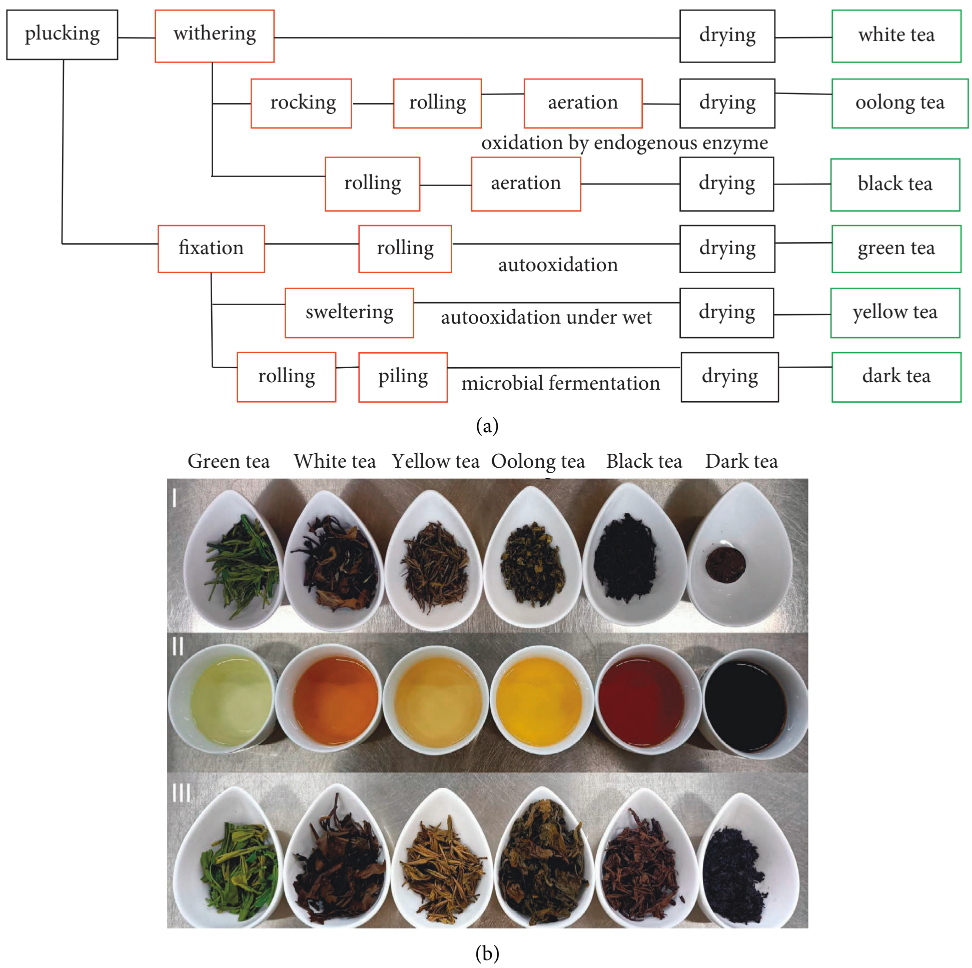

Figure 1: Manufacture processes of six major types of tea (a) and the dried leaves (b) (I), tea soups (b-II), and tea leaves after imbibed with hot water at 90°C (b-III) of six major types of tea.

oolong tea is "semioxidized" tea, and black tea is "fully oxidized" tea [10]. White tea is obtained by only withering and drying, as more prolonged withering leads to a severe water deficiency that induces membrane disintegration and polyphenol oxidization by endogenous enzymes. In addition, white tea can be stored for a long time, during which chemical reactions such as catechin and amino acid oxidation can occur that induce better flavor and taste (more sweetness and smoothness) and more health benefits [11–13].

The objective of our study was to "improve" (Figure 2) the abovementioned "traditional" method by adding an ethyl acetate extraction step between liquid tea production and n-butanol extraction. Moreover, our method's new equations for the TB content (%) of the six major types of tea extraction had different absorbance values at 380 nm (A_{380}) and slope compared to black tea analyses by the traditional method.

2. Materials and Methods

2.1. Samples. The six major types of tea samples obtained are listed in Table 1. All were purchased from local tea markets located in Anxi, Fujian Province, Dali, Yunnan Province, and Chongqing City, China. Each tea sample was ground into tea powder using a grinder (Joyoung Co., Shandong, China) and then stored in sealed cans until analysis.

2.2. Preparation of Liquid Tea Samples. Boiling distilled and deionized water (d.d. H_2O; 125 mL) was added to 3.0 grams of tea powder in a 250 mL conical flask and shaken in a water bath at 90°C for 10 min. The liquid tea was then centrifuged at 1,800 × *g* for 10 min at 4°C. The supernatant was diluted to 125 mL with d.d. H_2O and then divided into two aliquots, one for TB extraction by the traditional method and the other by our improved method (Figure 2).

2.3. TB Extraction. The traditional method was based on Yao et al. [33] and Roberts and Smith [35, 36], with a few modifications. Briefly, 25 mL of tea solution was extracted with the same volume of *n*-butanol (Xilong Scientific Co., Guangdong, China) and shaken for 3 min. The lower layer of the solution was then centrifuged at 1,800 × g for 10 min at 4°C. Two mL of the supernatant was then mixed with 2 mL of saturated (10.2%) oxalic acid (SINOPHARM Co., Shanghai, China) and 6 mL of d.d. H_2O was then added, followed by dilution to 25 mL with 95% (v/v) ethanol (SINOPHARM Co., Shanghai, China). A_{380} values of the above solutions were measured using a UV-1750 spectrophotometer (Shimadzu, Japan), and 95% (v/v) ethanol was used as a blank. TB content (%) = $[7.06 \times 2 \times A_{380} \times 100\%]/[w \times 1/3 \times (1\text{-M})] = [21.18 \times 2 \times A_{380} \times 100\%]/w \times (1\text{-M})$, where "$w$" is the weight (gram) of the tea sample, "M" is the moisture content (%) of the tea sample, and 21.18 is the inverse slope of $A_{380.}$

The improved method with TB concentrations from TB extracted samples was modified from the abovementioned traditional method. Mainly, 35 mL of ethyl acetate (SINOPHARM Co., Shanghai, China) was added to samples of the six tea types (35 mL) in a 125 mL separating funnel and shaken for 3 min. After discarding the upper layer, the lower layer (25 mL) was then extracted with 25 mL of *n*-butanol (Xilong Scientific Co., Guangdong, China) and shaken for 3 min; the lower layer solution was used for centrifugation at 1,800 × g for 10 min at 4°C. The A_{380} detections of the solutions were done the same way as in the abovementioned traditional method, and TB contents (%) were determined by the six equations for the improved method. TB in the six tea types was extracted by the improved method and then freeze-dried. Freeze-dried samples were dissolved in d.d. H_2O, various concentrations of TB solution (2 mL) were mixed with 2 mL of saturated oxalic acid, 6 mL of d.d. H_2O was added, and the samples were then diluted to 25 mL with 95% (v/v) ethanol. Samples were then measured at A_{380}.

2.4. Moisture. Moisture in the tea powder was measured using a vacuum oven according to an international standard method (ISO1573 (BS6049-2), 1980).

2.5. Data Analysis. Paired data with A_{380} values of both traditional and improved methods were subjected to paired *t*-tests using Microsoft Excel 2019. TB contents (%) are presented as mean values ± standard deviations (SD) of twelve independent sets of experiments with similar results. Paired *t*-tests were calculated with high significance at $p \leq 0.01$using SPSS version 23.0 (SPSS, Chicago, USA). Linear equations were established by regression analysis between A_{380} measurements and TB concentrations of the six tea types using SigmaPlot ver. 12.5 (SYSTAT Software, San Jose, CA).

3. Results and Discussion

3.1. Comparisons of TB Extractions between Traditional and Improved Methods. Figure 3 illustrates A_{380} values from TB solutions extracted by traditional and improved methods. Readings from the improved method were significantly lower (around 80–90%) compared to the traditional method in all six tea types, indicating that ethyl acetate extraction removes TFs and portions of TRs from liquid tea [33] and decreases TB A_{380} values. TB compositions were different between these extraction methods, and revised parameters for the improved method should therefore be established.

After plucking, yellow, green, and dark tea leaves are first fixed by steaming or pan-frying (Figure 1(a)) to inactivate all endogenous enzymes. Green tea is produced after rolling and drying. Yellow tea is obtained when tea leaves are kept wet and under high temperatures for 6 to 12 h (Figure 1(a)) between fixing and drying, during which chlorophylls are degraded and polyphenols are auto-oxidized. Dark tea is harvested after leaves have been kept wet and microorganisms grow on their surfaces for many days between fixing and drying (Figure 1(a)). During this piling process, microbes (mainly *Aspergillus fumigatus*, *Aspergillus Niger,* and *Saccharomyces cerevisiae*) secrete many enzymes to induce polyphenol oxidation, cell wall degradation, and fermentation [14–17]. TB content increases during dark tea processing [18], and the leaves become dark brown (Figure 1(b)).

Similar plant secondary metabolites can reduce the risk of age-related chronic diseases and promote health benefits [19, 20]. TBs have physiological functions such as reducing blood lipid and blood sugar levels [21–23]; controlling of diabetes mellitus [24]; attenuation of hypercholesterolemia [25, 26]; reducing serum levels of total cholesterol, low-density cholesterol, and triglycerides [27]; and osteoclastogenesis suppression and prevention of bone loss [28], together with inhibition of cell cycling and tumor cell growth [29]. In addition, TB content is a positive parameter in evaluating fragrance and flavor in dark tea and white tea [9, 18, 30, 31]. Zhu et al. [32] and Cheng et al. [18] reported that stringent taste levels were decreased and stale and fungal aromas increased with TB content. Furthermore, dark tea and white tea leaves can be stored for a long time, and longer storage times result in higher TB content [14]. In contrast, during storage, an increased TB content worsens tea quality in both black tea and oolong tea due to the loss of aroma and sweetness molecules [5, 7, 33]. Therefore, TB content may be an objective measure of tea quality [34]. Yao et al. [33] combined three methods [35–37] to analyze TF, TR, and TB contents and constructed a "traditional" method of TB extraction (Figure 2). However, TBs are defined as a variety of water-soluble compounds but are not dissolved in n-butanol and ethyl acetate [22, 27]. In the traditional method, without ethyl acetate extraction, TFs or TRs would be retained in the TB layer solution and thus affect the accuracy of TB content analysis, because A_{380} is detected from TF, TR, and TB solutions. In addition, the TB equation was based on black tea samples extracted from the traditional method [33, 35, 36]. Whether the slop parameter of the black tea equation is the same as other types of tea remains unknown.

3.2. Equations Established for the Improved Method. The traditional method's empirical equation for determining TB content (%) is $[21.18 \times 2 \times A_{380} \times 100\%]/w\ (1\text{-M})$, where "$w$" stands for the weight (in grams) of tea sample powder and 21.18 is the inverse slope of A_{380} based on black tea [33, 35, 36]. In our study, six major types of tea were extracted by the improved method, their solutions were freeze-dried, and TB powder was collected. After TB

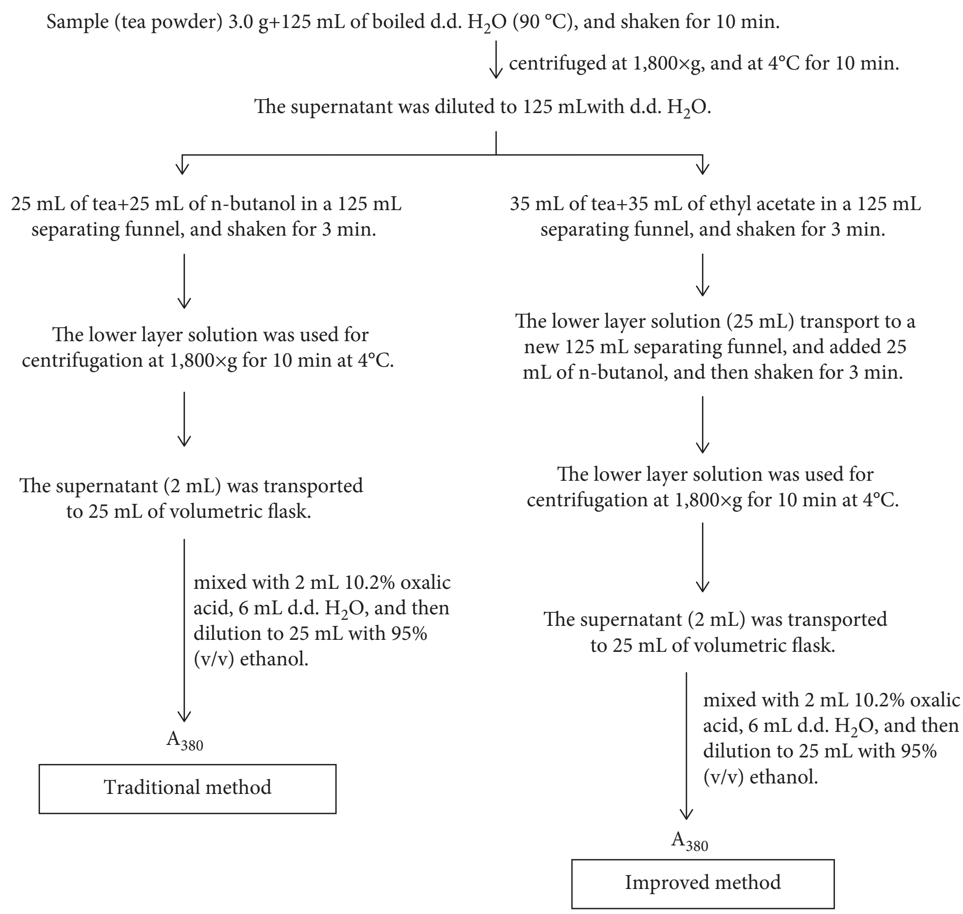

FIGURE 2: Flow chart of the traditional and improved methods of TB extractions.

TABLE 1: List of six major types of tea sample and producing location.

Type of tea	Tea sample and producing location
Black tea	Lapsang Souchong, produced in Fujian province, China Yunnan black tea, produced in Yunnan province, China
Oolong tea	Wuyi Dahongpao, produced in Fujian province, China Anxi Tieguanyin, produced in Fujian province, China
Dark tea	Ripened Pu-Erh teas, produced in Yunnan province, China, storage for 4 years Ripened Pu-Erh caked tea, produced in Yunnan province, China, storage for 4 years
White tea	Gong Mei, produced in Fujian province, China, storage for 4 years Baimudan, produced in Fujian province, China, storage for 4 years
Yellow tea	Huoshan Huangya, produced in Anhui province, China
Green tea	Longjing, produced in Zhejiang province, China Jinyun Maofeng, produced in Chongqing city, China

powders were dissolved in d.d. H_2O, different A_{380} values were observed from the samples. Figure 4 shows that A_{380} values were significantly ($p < 0.0001$) and positively correlated with the TB concentrations of green tea ($r = 0.9963$, $R^2 = 0.9926$), yellow tea ($r = 0.9961$, $R^2 = 0.9922$), oolong tea ($r = 0.9977$, $R^2 = 0.9954$), black tea ($r = 0.9987$, $R^2 = 0.9975$), white tea ($r = 0.9973$, $R^2 = 0.9947$), and dark tea ($r = 0.9975$, $R^2 = 0.9950$). Regression equation slopes for dark tea, white tea, black tea, oolong tea, yellow tea, and green tea were 0.0704, 0.0475, 0.0381, 0.0350, 0.0166, and 0.0160, respectively. Furthermore, the inverse slopes of dark tea, white tea, black tea, oolong tea, yellow tea, and green tea were 14.205, 21.053, 26.247, 28.571, 60.241, and 62.5, respectively. The equations for the six tea types using the improved method were as follows: dark tea:

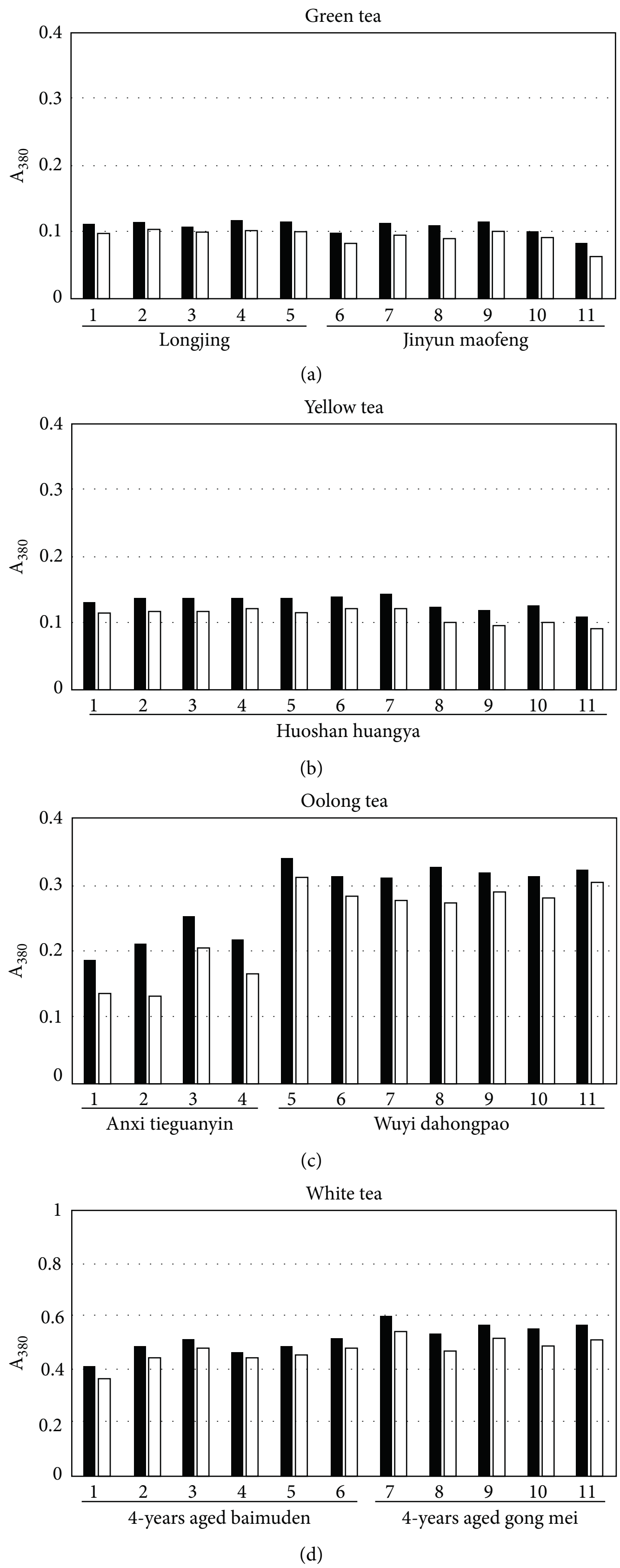

Figure 3: Continued.

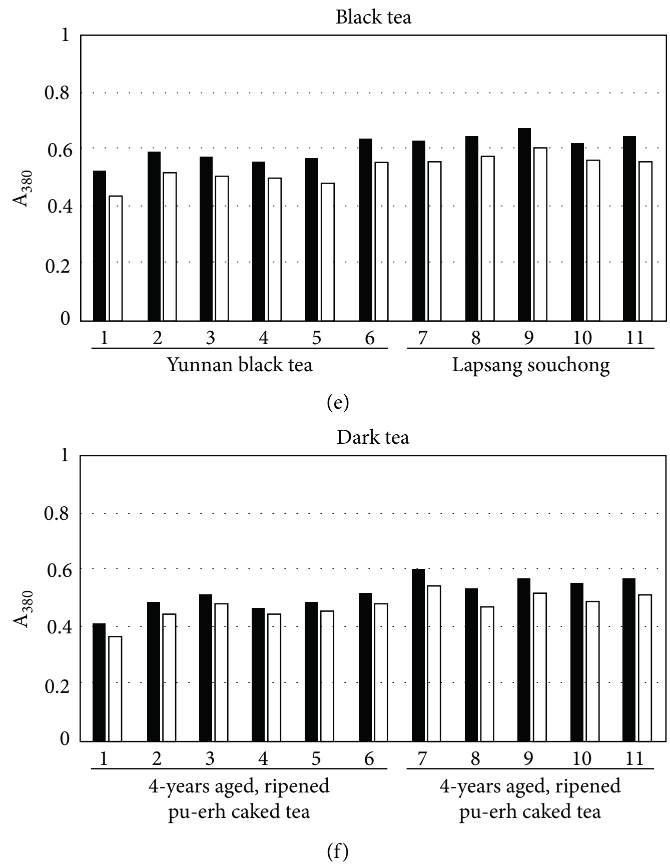

FIGURE 3: Comparisons of A_{380} values with TB extractions by the traditional method (black bar) and the improved method (white bar) of six major types of tea: green tea (a), yellow tea (b), oolong tea (c), white tea (d), black tea (e), and dark tea (f). Test numbers of tea samples (1~11) in each major type of tea: (a) 1–5, Longjing, and 6–11, Jinyun Maofeng in green tea. (b) 1–11, Huoshan Huangya in yellow tea. (c) 1–4, Anxi Tieguanyin, and 5–11, Wuyi Dahongpao in oolong tea. (d) 1–6, four-years-old Baimudan, and 7–11, four-years-old Gong-Mei white tea. (e) 1–6, Yunnan black tea, and 7–11, Lapsang Souchong black tea. (f) 1–6, four-years-old, ripened Pu-Erh teas, and 7–11, four-years-old, ripened Pu-Erh caked dark tea.

$[14.205 \times 2 \times A_{380} \times 100\%]/w \times (1\text{-M})$, white tea: $[21.053 \times A_{380} \times 100\%]/w \times (1\text{-M})$, black tea: $[26.247 \times 2 \times A_{380} \times 100\%]/w \times (1\text{-M})$, oolong tea: $[28.571 \times 2 \times A_{380} \times 100\%]/w \times (1\text{-M})$, yellow tea: $[60.241 \times 2 \times A_{380} \times 100\%]/w \times (1\text{-M})$, and green tea: $[62.5 \times 2 \times A_{380} \times 100\%]/w \times (1\text{-M})$.

In those empirical equations, "M" means the tea sample's moisture content (%) and "w" stands for the weight (in grams) of tea sample powder. Figure 4 shows that two types of aerated tea (black tea and oolong tea) had similar variations, but green tea and yellow tea almost completely overlapped, indicating that the TB compositions were similar in these two types of tea. Wang et al. [38] demonstrated that green tea and yellow tea had similar chemical components according to metabolome analysis. After high-temperature treatments in fixation and drying processes during storage, isomer flavanols and chlorophyll metabolites (e.g., pheophytins) were produced in the autooxidation process, and the color of tea leaves turned olive brown [39–43]. The slope of white tea was higher than that of black tea and lower than that of dark tea (Figure 4), implying that the TB composition in white tea is notably different from that in dark tea and black tea. Although liquid chromatography-mass spectrometry analysis was used to determine the chemical composition in white tea after long-term storage [11, 12, 44], the TB composition still remained unknown. In addition, the TB of dark tea contains some fungi-specific metabolites detected by gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry-based metabolomics [18, 23, 32, 45–49].

3.3. Comparison of TB Content between Traditional and Improved Methods in Tea Extractions. TBs were extracted by both traditional and improved methods, and calculations were made with appropriate equations at A_{380}. Figure 5 shows that the TB contents of these two methods were significantly different ($p < 0.01$) in all types of liquid teas.

We repeated many studies by using the traditional extraction method and found that dark tea had the highest TB content (10%~14%) of the six tea types [14, 15, 50, 51], followed by black tea (7%~9%) [14, 50, 52], and ranges of 2%~3.5% in green tea and yellow tea [50, 52]. The improved method showed that black tea had the highest TB content (7.97%~11.19%, average 9.75%), and oolong tea ranged

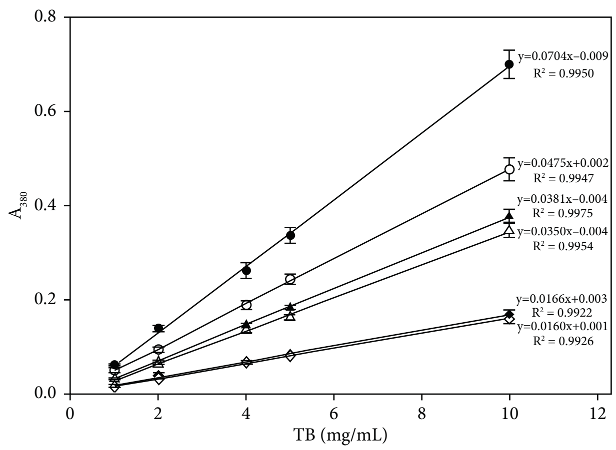

Figure 4: The A_{380} variations of TB concentrations extracted by the improved method from six major types of tea (◇: green tea, ◆: yellow tea, △: oolong tea, ▲: black tea, ○: white tea, and ●: dark tea). Linear equations were established by regression analysis of linear relationships between TB concentrations and A_{380} measurements. Each data point represents the mean ± SD of three independent measurements.

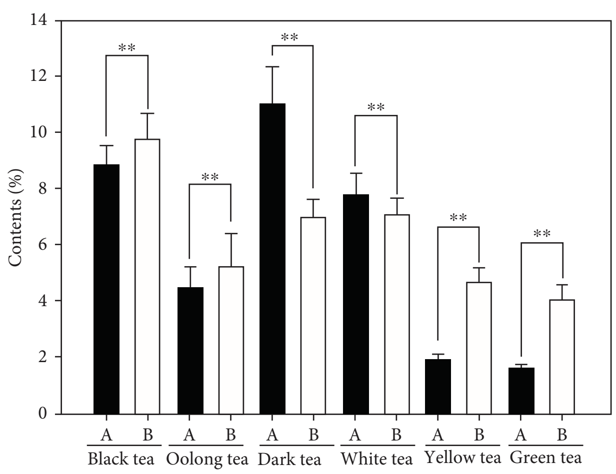

Figure 5: Paired comparisons of TB contents (%) of six major types of tea extracts between the traditional method (a) and improved method (b). Each bar represents the mean ± SD of twelve independent measurements. Asterisk (∗ ∗) indicates high significance at $p \leq 0.01$.

2.65%~6.23% with an average of 5.25%. Black tea and oolong tea were oxidized by endogenous enzymes in a longer aeration process and had higher TB contents. The TB contents of white tea ranged from 5.35% to 8.02%, averaging 7.03%, suggesting that four years of storage was sufficient to transfer high levels of TB content in the leaves. The TB contents of dark tea ranged from 5.66 to 7.66%, averaging 6.96%, while green tea and yellow tea ranged from 2.63 to 4.46% with an average of 4.03% and 3.87 to 5.2% with an average of 4.61%, respectively, indicating that their values differed significantly from levels derived from the traditional extraction method. Dark tea had the highest A_{380} of the six tea types (Figure 3); however, high slop in TB concentrations (Figure 4) resulted in TB content not being as high as with the traditional method (Figure 5). TB composition is formed during tea processing, and the aeration procedure is one of the most important steps in black tea and oolong tea processing, while it does not occur in other teas. Therefore, different equations are proposed to calculate TB content in different types of tea, especially dark tea, yellow tea, and green tea.

4. Conclusion

This study provided an improved method for the analysis of TB content (%) from six major types of tea. This method decreases the A_{380} values of TBs with differing TB absorbance capabilities in six major types of tea. Six equations have been developed to analyze TB content (%) in those six tea types extracted by an improved method, and the ranges of TB content (%) showed significant differences compared to the traditional method.

Abbreviations

SD: Standard deviation

Data Availability

The data used to support this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors' Contributions

T-C L. designed the experiments; T-C L. and Q-N Z. collected the sample, conducted experiments, and analyzed the data. T-C L. and K-H L. wrote the article; T-C L., Q-N Z., H-L H., P-Y L., J-Y Z., C- K., and Y-J L. collected the sample and investigated the study; T-H Ko projected-ministration and reviewed and edited the manuscript.

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Supplementary Materials

Supplementary Figure 1: the simplified scheme of relationship among TF, TR, and TB. (*Supplementary Materials*)

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