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Development of a visual test kit for estimation of total polyphenols in tea

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Abstract

Regarding the tea quality, the International Organization for Standardization (ISO) has established recently the levels of minimum concentrations of total polyphenols in tea. Because of this standard, the commercial exploitation of tea demands the investigation of the levels of total polyphenols. However, it is quite difficult for most producers and traders to implement because this test is laborious, time consuming, required instruments and trained technicians. Therefore, a visual test kit for the estimation of total polyphenols was systematically developed and evaluated. The estimation was accomplished by reacting tea extract with Folin–Ciocalteu reagent in alkaline medium to produce a colored compound that was measured visually using a color strip. The results obtained demonstrated that the test kit method was simple, rapid, reliable and comparable with that of the standard method. It can be stated that the test kit is suitable for the estimating of total polyphenols in tea. Since no special equipment is required, the test can be performed at the production site or the tea market even by tea processing workers or tea traders.

Introduction

Tea (*Camellia sinensis* L.) is the most popular beverage in the world. Its popularity is attributed to its sensory properties and potential health benefits. Teas can be divided into three categories based on the degree of fermentations: green tea (unfermented), oolong tea (partially fermented), and black tea (fully fermented). Green tea is heated to avoid enzymatic oxidation in fermentation process. Oolong tea is semi-fermented to permit a partial level of enzymatic oxidation. Black tea is the most thoroughly oxidized enzymatically. The most important bioactive compounds in tea, which are of considerable pharmacological significance, are polyphenols. Increased public awareness of the health protective characteristics of tea are generally considered to be associated with the high polyphenol content of tea products. The assessment of total phenolic content has gained enormous attention in the last few years, especially in tea products. Therefore, the International Organization for Standardization (ISO) has proposed recently the standards of total phenolic content in tea for ensuring marketing requirements. Minimum concentrations of total polyphenols (TP) for black (ISO, 2011a) and green tea (ISO, 2011b) were 9 and 11 GAE g/100 g DW, respectively. Because of these requirements, the future commercial exploitation of tea as a health food will demand the investigation of the levels of polyphenols in different tea brands in addition to the necessary evidence of efficacy and regulatory compliance.

Many methods have been developed for quantification of total phenolic content in foods. Most of the available methods are based on the reaction of phenolic compounds with a colorimetric reagent, thus allowing their measurement in the visible region of the spectra (Robards and Antolovich 1997; Escarpa and Gonzalez 2001). Among these methods, the Folin-Ciocalteu (FC) assay is frequently applied (Singleton and Rossi 1965; Singleton *et al*., 1999), and recent studies have shown that total polyphenols determined by this method can be correlated to antioxidant activity determined by different methods ($ABTS^{+\bullet}$ and $DPPH^{\bullet}$ assays, for instance) (Roginsky and Lissi 2005). For this reason, the FC assay has been proposed used as a method for the routine quality control and measurement of antioxidant capacity of food products. The method described by ISO standard 14502-1 (ISO, 2005) has been established as a standardized method for use in the routine quality control and measurement of tea products. This standard is an analytical tool widely used to determine the phenolic compounds for both black and green teas. In this method, the TP is determined spectrophotometrically by a reaction with the FC reagent, a mixture of phosphomolybdic/phosphotungstic acid complexes as oxidants which on reduction by readily oxidized phenolic hydroxyl groups yields a blue color with an absorption maximum around 765 nm (Singleton *et al*., 1999). The blue product is proportional to the total quantity of phenolic compounds originally

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present. The gallic acid is recommended and widely used as a calibration standard (Prior *et al*., 2005) due to its satisfactory solubility, adequate stability and low price (Chun and Kim, 2004). The results are usually reported as gallic acid equivalent (GAE). The method is available for routine quantitative estimation of TP. But in developing countries, this method is quite expensive because the reagents have to be imported. Moreover, it is too difficult for most producers and traders to implement because this test is laborious, time consuming, required lab set-ups, required instruments and trained technicians to conduct tests. A new quantitative estimation of TP should be developed in order to provide simple, rapid and inexpensive analytical tools for tea industry.

The aim of this study was to develop a simple and fast test kit for estimation of total polyphenols in commercial tea products. This developed test kit could be used for tea producers to approximate the TP in tea factories. In addition, it is suitable for tea trader to use the results obtained from the test kit as a guideline in tea trading.

Materials and Methods

Tea samples

Tea samples used for validation were purchased from local supermarket in Thailand in 2011. They include green, oolong and black tea. All tea samples were carried out in triplicate.

Chemicals and reagents

Five FC reagents were purchased from Fluka, Merk, BDH, Sigma and Carlo Erba. Gallic acid was purchased from Fluka (Buchs, Switzerland). Anhydrous sodium carbonate was purchased from Merck (Darmstadt, Germany). All reagents for synthesis of Folin–Ciocalteu reagents, including sodium molybdate, sodium tungstate, lithium sulfate, hydrochloric acid, ortho- phosphoric acid and bromine were purchased from Carlo Erba (Italy).

Development of a test kit

The FC used in this study was synthesized in the laboratory. In brief, 10 g sodium tungstate and 2.5 g sodium molybdate were dissolved in 70 ml distilled water. Then, 5 ml 85% phosphoric acid and 10 ml concentrated hydrochloric acid were added, and the solution mixture was refluxed. After 10 h, 15 g lithium sulfate, 5 ml water and 1 drop bromine were added and continuously refluxed for 15 min. The solution was cooled to room temperature, and the volume was brought to 100 ml with distilled water. A synthesized FC was used to determine TP of known standards and tea samples using ISO 14052-1 standard method (ISO, 2005). The results were compared with that obtained from 5 commercial FCs. Studies of chemical aspects concerning the amount of FC, reaction time, and Na_2CO_3 concentration were carried out. Long-term stabilities of the reagents in the test kit were also investigated. The color strip was then developed and validated in order to approximately determine the TP by human eye perception. A prototype test kit was initially evaluated and developed in the laboratory with known standards and samples. A reliability of the test kit was evaluated by assaying the known standards and samples in order to check whether the tests pass or not.

Standard procedure for determination of total polyphenols

Tea samples were ground and 2 g, weighed to the nearest 0.001 g, of ground tea were extracted with 200 ml of boiling distilled water at a temperature of 95°C. The extraction mixture was constantly stirred with a magnetic stirrer. After 10 min, the extraction mixture was filtered through a filter paper (Whatman No. 4). The residue was washed with distilled water (3x10 ml). The tea solution was cooled to room temperature and adjusted to 250 ml with distilled water. The TP was determined by spectrophotometry, using gallic acid as a standard, according to ISO 14052-1 standard method (ISO, 2005). In brief, sample extracts were diluted 50-fold with distilled water and 1.0 ml portions of the diluted solution were transferred in duplicate to separate tubes containing 5.0 ml of 10%v/v dilution of FC reagent in water. Then, 4.0 ml of sodium carbonate solution (7.5% w/v) was added. The tubes were then left to stand at room temperature for 60 min before absorbance at 765 nm was measured. The concentration of polyphenols in the diluted extract was derived from a standard curve of gallic acid ranging from 10-100 µg/ml and multiplied by the dilution factor to obtain µg/ml of the original extract. This value was then multiplied by total volume of extract and divided by weight of sample. This gallic acid equivalents value (GAE) was further multiplied by 100 to directly convert to GAE g/100 g samples. All tests were performed in triplicate.

Test kit procedure for determination of total polyphenols

Determination of TP was carried out as a test kit protocol modified from ISO 14052-1 standard method. In brief, tea samples were ground, and 1 spoon (~0.2 g) of ground tea were extracted with boiling distilled water (20 ml) in an extraction bottle.

The mixture was shaken manually by hand. After 5 min, 2 ml of the solution was diluted with distilled water (100 ml) in a dilution bottle. The diluted solution (0.5) ml was transferred into a test tube containing 3 drops of synthesized FC (FC-MFU) and 1 ml of MFU1 (distilled water). Then, 1.0 ml of MFU2 (15%w/v Na_2CO_3) was added and well mixed. The tubes were then left to stand at room temperature for 10 min before the color intensity was compared with the color strip. The concentration of polyphenols in tea samples was derived from a color strip of gallic acid, ranging from 0 to 25 GAE g/100 g sample. All tests were performed in triplicate.

Statistical analysis

Results were analyzed statistically using SPSS 16.0 for Windows to determine mean values and standard deviations of at least three experiments. ANOVA with a significance level of $P < 0.05$ was considered significant differences. The test kit was initially evaluated and developed in the laboratory with known standards and samples. Student's t-test was applied to test significant difference between the means obtained by test kit and standard method.

Results and Discussion

Development of a test kit

The aim of this study was to develop a simple and fast visual test kit for estimation of TP in teas. The chemical system chosen was the classical reaction between FC reagent and phenolic compounds under alkali medium. Quantitative laboratory analysis of TP is generally done using spectrophotometry. Although, this technique provided accurate quantification of TP and was successful in TP determination; it did not align with our goal. In addition, the primary purpose of the test kit centered on the ability of a tea processing worker and/or a tea trader to perform the test, spectrophotometry was not considered in this research. Therefore, different systematic steps were studied in order to achieve our objectives. First, the chemical reaction of the test kit procedure was improved. Then, a prototype test kit was produced and the use of test kit was evaluated.

Study of chemical aspects

Initial studies were carried out in order to develop an appropriate visual test kit using gallic acid as a standard. In this study, the FC was synthesized in a laboratory, which was named as FC-MFU. The first experiment was performed by checking the efficiency of FC-MFU in determination of TP. This was done by examining the gallic acid standard and tea samples using ISO standard method. The results obtained from FC-MFU were not significantly different from 5 commercial FCs (data not shown). Therefore, FC-MFU was used to develop a test kit.

The test kit developed in this study was modified from ISO 14052-1 standard method (ISO, 2005). A significant change to the ISO method was the decreased volume of the reagents. In preliminary assay, the FC-MFU was added to 1.0 ml of distilled water. Then 0.5 ml of sample extracts or standards (10-100 µg/ml) were added. Later, 1.0 ml of 7.5%w/v Na_2CO_3 was added and thoroughly mixed. The tubes were then left to stand at room temperature for 60 min before absorbance at 765 nm was measured. The result was derived from a standard curve of gallic acid (10-100 µg/ml).

Studies of chemical aspects concerning the amount of FC, reaction time, and Na_2CO_3 concentration were carried out. A preliminary study on the effect of the amount of FC in the reaction mixture was carried out. A gallic acid standard (50 µg/ml, 0.5 ml) was mixed with 1.0 ml of distilled water. This mixture was treated with increasing amounts of FC (1, 2, 3 and 4 drops). After 60 min, the absorbance of the reaction color was measured. The result was derived from a standard curve of gallic acid (10-100 µg/ml). The mean concentration obtained was 52.94, 51.59, 50.06 and 50.13 for 1, 2, 3 and 4 drops of the FC, respectively. Thus, 3 drops of FC-MFU was chosen for the next experiment as it provided the highest accuracy. To evaluate the effect of Na_2CO_3 concentration, a similar experiment to that described above was performed. For this, the Na_2CO_3 concentrations were varied (7.5, 10, 12.5, 15, 17.5 and 20 %w/v). The absorbance of reaction color was recorded and the pH of the reaction was recorded at intervals. It is clear from Table 1 that the higher alkalinity of the reaction (higher concentration of Na_2CO_3), the more rapidly production of reaction color takes place, conversely the higher alkalinity, the shorter is the length of the plateau at the maximum absorbance. It is important to have enough but not excessive alkalinity because it affects the stability of the complex formed (Singleton *et al*., 1999). At 15%w/v, a reasonable balance is obtained and the reaction takes under 10 minutes to reach its maximum color, while the plateau at the maximum lasts around 25-30 minutes. The reaction color is stable for 30 minutes. Thus, the concentration at 15%w/v Na_2CO_3 was chosen for the test kit.

Another investigation was the stability of the reagents. All reagents were stored at room temperature (~25-35˚C) and used to analyzed a know concentration of a freshly prepared gallic acid (50 µg/ml). Figure 1 shows that the result of TP assay remains 98% of the

Table 1. Stability of absorbance at various concentrations of sodium carbonate

Concentration of Na_2CO_3 (%w/v)	Approximate reaction pH (0-60 min)	Approximate time to read maximum (min)	Approximate length of plateau at maximum (min)
7.5	11.53-11.14	50-60	>60
10.0	11.64-11.33	40-45	>50
12.5	11.85-11.55	20-24	40-45
15.0	11.95-11.80	6-8	25-30
17.5	12.05-11.92	4-5	10-15
20	12.20-11.97	2-3	6-9

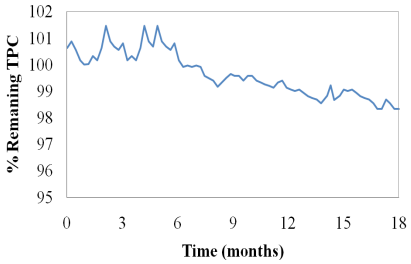

Figure 1. The percentage of remaining TPC obtained by assaying a freshly prepared gallic acid standard (60 µg/ml) with the reagents over 18 months of the experiment

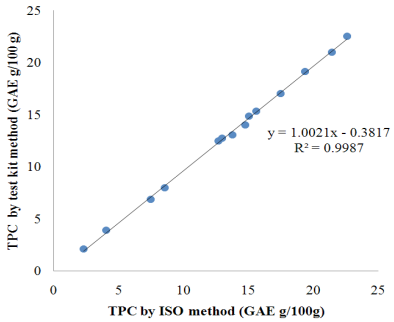

Figure 2. Correlation between ISO method and test kit method

origin after all reagents were stored for 18 months. It indicated that all reagents were stable in solution with a minimum 18 month shelf life.

The test kit developed contained 3 reagents, including FC-MFU (synthesized FC), MFU-1 (distilled water) and MFU-2 (15%w/v Na_2CO_3). A prototype test kit was produced and used to analyzed spectrometrically using standard and test kit methods. A comparison of the overall results of TP determinations by the test kit and the ISO method was shown in Figure 2. The estimation of TP by the test kit correlated well with estimations based on ISO standard method, with correlation coefficient (r^2) being high at 0.9987. Furthermore, when a paired t-test was performed on the data obtained for all samples, it indicated no significant difference for the mean TP obtained by the two methods.

Developments of a color strip

To develop the visual test kit without the use of spectrophotometer, a color strip was developed. A series of standard at concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml was firstly assayed as the test kit method. Then the colors

Table 2. Summarized results of visual reading of testers

Test	Identification	Number of tests	Number of results			
			Pass (%)	Pass in acceptable range (%)	Total pass (%)	Failed (%)
1	9 standards	126	115 (91)	9 (7)	124 (98)	2 (2)
2	6 samples	84	75 (89)	6 (7)	81 (96)	3 (4)
3	16 spiked samples	224	186 (83)	22 (10)	208 (93)	16 (7)
4	5 samples	245	197 (80)	48 (20)	245 (100)	0 (0)

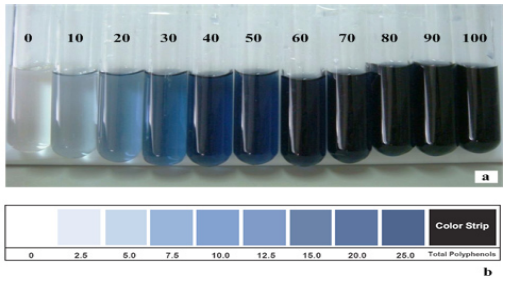

Figure 3. A color obtained from the reaction between the FC and 0-100 µg/ml gallic acid standards (a) and a developed color strip (b)

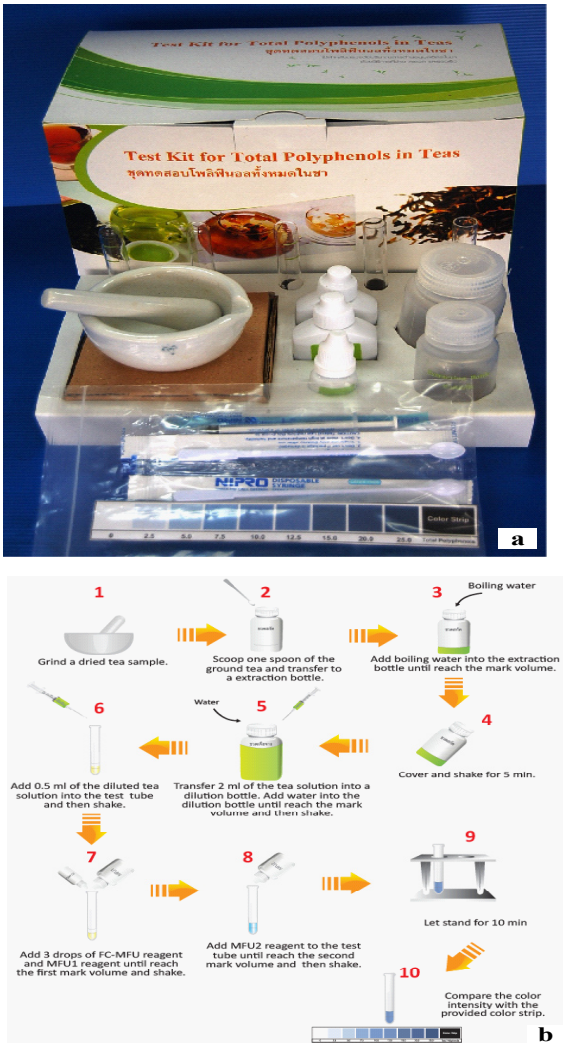

Figure 4. A prototype test kit (a) and a test kit method (b)

of each concentration were observed visually by human eye perception. We found that human eye perception was indistinguishable the color intensity

more than the concentrations of 50 µg/ml (Figure 3a). Therefore, 0-50 µg/ml was chosen for making the color stip. This concentration range was then assessed for optimization. The concentrations of a gallic acid standard used for making the color strip eventually developed for visual reading were 0, 5, 10, 15, 20, 25, 30, 40 and 50 µg/ml. These concentrations corresponded to 0, 2.5, 5, 7.5, 10, 12.5, 15, 20 and 25 GAE g/100 g samples, respectively (Figure 3b).

Materials of a test kit

The goal was to provide prepared materials in the test kit which allows testers to quickly analyze TP without the use of analytical instrumentation. Therefore, the test kit would provide all necessary components for TP estimation, including 3 reagents, accessories, a manual and a color chart displaying colors representative of the TP (Figure 4). By using the materials provided of a test kit, a tester can grind, extract the phenolic compounds in teas and add the extract to a test tube containing the necessary reagents. After 10 min of reaction, the color in the test tube is compared to a color chart.

Reliability of a prototype test kit

In order to demonstrate the applicability of the test, the evaluation was done with 4 tests, and the results were shown in Table 2. The test kit was firstly validated by using gallic acid standards (test 1) and then applied to the estimation of different real samples (test 2-4). The analysis was performed as a test kit procedure. After complete reactions, the results were read individually by fourteen testers in order to prevent an influence on the judging. The visual interpretations are as follows: when testers read correct values as expected, results are "pass"; when testers read deviated values (less or more one error level), results are "pass in acceptable range"; when testers read wrong values, results are "failed".

For test 1, a series of standard at concentrations of 0, 5, 10, 15, 20, 25, 30, 40 and 50 µg/ml was tested (total 126 tests). For test 2, six tea samples with known concentrations of TP (determined by ISO method) were then tested (total 84 tests). Table 2 shows that the test kit has fairly good results. The numbers of total pass were 124 (98%) and 81 (96%) for standards (test 1) and samples (test 2), respectively. The accuracy (test 3) was then evaluated by spiking four tea samples with four different concentrations (5, 10, 15 and 20 GAE g/100 g) of the standard (total 224 tests). It was observed that the accuracy was good with 208 (93%) of total pass. The consistency in visual judging by the testers was checked in test 4. This was evaluated by estimation of TP in five tea samples. Each sample was tested seven times and the results were read individually by seven testers (total 245 tests). Precision was excellent with 245 (100%) of total pass. In this study, it needs to be emphasized that all testers did not receive training in the reading of results from the color strip. They all had very little experience in the color interpretation. The reading results from Table 2 clearly show that the test kit has good results for all tests performed.

Although, the visual test kit provides fairly good results in testing with tea, it has limitations for using only in dried teas due to the interference reacting with the FC. These interferences were contributed by non-phenolic antioxidants and reducing substances (ascorbic acid, glucose, fructose, sulphites) that are common food additives or are naturally present in fruits and vegetables. Amino acids (tyrosine, tryptophan) and proteins containing these amino acids also formed a blue color with the FC (Peterson, 1979, 1983). Moreover, Cicco *et al.* (2009) and Cicco and Lattanzio (2011) were the first to describe the interference of alcohol in FC reaction mixtures. They recommended that the final reaction mixtures should not exceed 4% alcohol by volume. These interferences make our test kit can be used only for dried tea. It is not appropriate to use with ready-to-drink, instant or others tea product added with additives.

In addition to the interferences, further improvement of this test kit is needed. Several issues need to be considered: i) knowledge of the testers about the test procedure; ii) training of testers for developing skills for proper color perception to minimize the human judgment errors; iii) checking the quality of testing reagents in different storing conditions; and iv) evaluating the satisfactory of users.

Conclusion

In summary, we have developed a visual test kit method for the estimation of total polyphenols in tea samples. The test kit was successfully applied to tea samples providing results that are comparable with the ISO method. The present test kit for the estimation of TP represents a suitable tool for tea producer and tea trader. However, further studies are needed. More data on the use of the visual test kit by testers are required. In addition, more robustness studies must be performed to validate the method developed in this study. If the data is determined to be significantly similar, the test kit can be developed in the future for use within the tea industry.

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