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Determination of Tannin in Green Tea Infusion by Flow-Injection Analysis Based on Quenching the Fluorescence of 3-Aminophthalate

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A flow-injection analytical system was developed to determine tannin content in green tea infusions. The flow-injection system is based on measuring the quenching effect of tannin on the fluorescence of 3-aminophthalate. Fluorophore was obtained by auto-oxidation of luminol during solution preparation. System performance was satisfactory for routine analysis (sample throughput $>20\ h^{-1}$; linear dynamic range for tannic acid, 0.005−0.3 mg/mL; linear dynamic range for green tea tannin, 0.02−1.0 mg/mL; CV < 3%). The flow-injection method is immune from interference by coexisting ascorbate in green tea infusion. Analytical results were verified by the ferrous tartrate method, the Japanese official analytical method.

KEYWORDS: Flow-injection; tannin; fluorescence; quenching; luminol; 3-aminophthalate; green tea

INTRODUCTION

Tannin is the major secondary metabolite of high-order plants, and these polyphenol-related chemicals are thought to be principal molecular defense mechanism against herbivores and viruses (*1*). Some herbivores have evolved or culturally adapted to the astringent taste of some tannins and developed species-specific habitual ingestion behaviors (*2*). Tea is perhaps the most obvious human example of such adaptation.

Therefore, tannin content is a reasonable and important parameter for evaluating tea quality and that of commercialized products. Several academic studies have identified positive physiological effects and health promotion characteristics of tea tannin (*3*). Consequently, a reliable and convenient method for quantifying tea tannin is urgently required for both quality control and health concerns.

The Folin method was adopted in the U.S. as the official method for analyzing tannin (*4*, *5*); however, this redox-based method is susceptible to interference from coexisting reducing ingredients (*6*). Ascorbate is among the most problematic reducing chemicals in fresh plant tissue; therefore, tannin content in unfermented green tea cannot be determined with the official U.S. method. Because unfermented green tea, *sencha*, is the most popular tea product in Japan, the ferrous tartrate method was chosen (*7*). The Japanese official method is based on complex-formation with tannin, and its analytical results are not affected by coexisting ascorbate content.

As rapid, economic, and versatile, many flow-injection methods are automated and commercialized (*8*). There is also a recent tendency to adopt flow-injection methods as official analytical methods. However, in a preliminary study by the authors, adapting the ferrous tartrate method to a flow-injection format caused precipitation inside the manifold; therefore, methods based on different mechanisms require investigation.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is a common and cheap chemiluminescence reagent (*9*, *10*). Cui et al. developed a sensitive flow-injection tannin assay based on inhibiting Cu^{2+}-catalyzed luminol chemiluminescence (*11*). Similar approaches have been applied for different analytical purposes (*12*). However, such oxidative chemiluminescence reactions are still affected by interference from reducing compounds, and the flow rate (>12 mL/min in the manifold in the study by Cui et al.) must be sufficiently high to immediately catch a decaying luminescence signal.

In the proposed approach, the fluorescence-quenching effect of tannin on oxidized luminal, 3-aminophthalate, was employed to quantify tannin content. Because a fluorescence signal is stable and very easy to handle with a flow-injection manifold, flow-rate can be tuned only by considering sample dispersion. More significantly, the fluorescence-quenching effect does not involve redox processes, a benefit for samples containing high ascorbate levels.

MATERIALS AND METHODS

Chemicals and Solutions. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), sodium bicarbonate, and potassium dihydrogen phosphate were purchased from Nacalai Tesque, Japan. Ammonium

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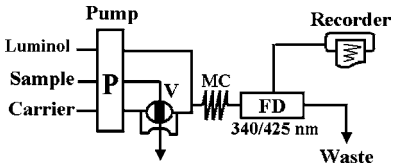

Figure 1. Schematic representation of the proposed FIA manifold. P, peristaltic pump; V, injection valve (20 μL); MC, mixing coil (20 cm); FD, fluorescence detector (excitation wavelength = 340 nm; emission wavelength = 425 nm; slit = 10 nm).

ferrous sulfate hexahydrate, potassium sodium (+)-tartrate tetrahydrate, and tannic acid were obtained from Wako Co., Japan. Sodium hydroxide was from Union Chemical Co., Taiwan. All chemicals were of analytical reagent grade and used as received.

Deionized water with conductivity <1 μS cm^{-1} was used to prepare buffers and solutions. Carbonate buffers were prepared by adjusting sodium bicarbonate aqueous solutions with sodium hydroxide to the desired pH levels. Phosphate buffer (0.1 M, pH 6.8) was prepared by dissolving 6.8 g of potassium dihydrogen phosphate and 1.0 g of sodium hydroxide with 500 mL of deionized water. Buffers were stored at ambient temperature. Luminol stock solution (25 mM) was prepared by dissolving solid luminol in 0.1 M carbonate buffer (pH 10.0) under mild sonication. Ferrous tartrate stock solution was developed by dissolving 0.1 g of ammonium ferrous sulfate hexahydrate and 0.5 g of potassium sodium (+)-tartrate tetrahydrate in 100 mL of phosphate buffer (0.1 M, pH 6.8). Standard tannin solutions and sample solutions were prepared/diluted with deionized water. These solutions were stored at 4 °C until they were used.

Flow-Injection Manifold. Conventional flow-injection tubing (silicon or Teflon tubes with 1 mm i.d.) and polypropylene connectors were used to assemble the system (**Figure 1**). The carrier (deionized water), reagent (luminol in 0.1 M carbonate buffer), and sample streams were driven (0.5−1.0 mL/min for each) with a signal-controllable three-channel peristaltic pump (SMP23S, Tokyo Rika Co., Japan). A 20-μL aliquot of sample solution was injected into the carrier stream and merged with the reagent stream. After mixing in a 20 cm coiled tube (the mixing coil), the resulting sample plug was delivered through a fluorometric detector (FP-1520, Jasco Co., Japan) to monitor fluorescence (λ_{ex} = 340 nm; λ_{em} = 425 nm; slit = 10 nm). Fluorescence intensity was converted to a voltage signal in the millivolt range, and the electric signal was attenuated to a suitable extent and recorded with a chart pen recorder.

Ferrous Tartrate Method. One aliquot (10 mL) of the ferrous tartrate stock solution was diluted with 5 aliquots (50 mL) of phosphate buffer (0.1 M, pH 6.8). Into a test tube, 200 μL of sample solution (tannin concentration = 0−2.5 mg/mL), 3.0 mL of diluted ferrous tartrate solution, and 2.5 mL of phosphate buffer were added sequentially. A_{550} was measured 10 min after the mixing.

Extraction of Tannin from Green Tea Leaves. In a beaker, 1.0 g of dried tealeaves were immersed in 200 mL of acetone/water solvent mixture (acetone:water = 9:1) for 30 min, and solvent in the filtrate (0.45 μm nitrocellulose filter) was evaporated completely with a rotary evaporator (*13*). The solid was stored at 4 °C.

RESULTS AND DISCUSSION

Fluorescence Scheme of 3-Aminophthalate. Figure 2 depicts the relationship between luminol chemiluminescence and 3-aminophthalate fluorescence. Because both processes share the same excited state, the chemiluminescence spectrum of luminol is similar to the emission spectrum of 3-aminophthalate with λ_{max} at 425 nm (*9*). Fluorescence of 3-aminophthalate had once been used by Chen for monitoring the acetylcholine releasing process of the synaptosomes from Japanese electric ray, *Narke japonica* (*14*).

Polyphenols were applied to enhance or inhibit luminol chemiluminescence (*10*); these chemicals can function as redox mediators accelerating oxidation of luminol and quenchers to facilitate the emissionless relaxation process of excited 3-aminophthalate. The latter effect will certainly reduce the fluorescence of 3-aminophthalate.

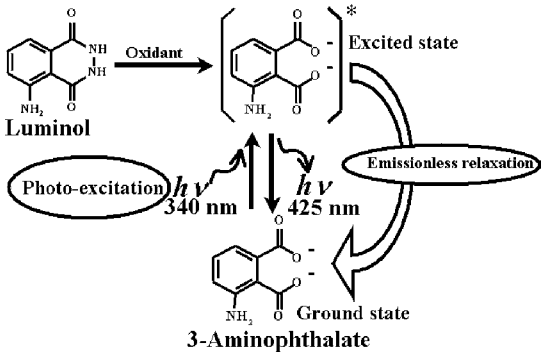

Figure 2. Fluorescence/chemiluminescence scheme of luminol and 3-aminophthalate.

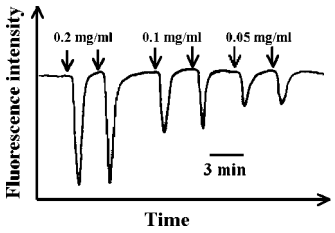

Figure 3. Dose-dependent FIAgram of tannic acid. Different concentrations of tannic acid were injected at the indicated times (the arrows). Flow rate, 0.5 mL/min; luminol concentration, 0.5 mM. The fluorescence intensity was not calibrated, and the ordinate is scaled as an arbitrary unit.

Dose-Dependent Fluorescence-Quenching Effect of Tannic Acid. The baseline fluorescence of the FIAgram (**Figure 3**) was that of 3-aminophthalate generated during the preparation and storage of the luminol solution. The fluorescence intensity of a freshly prepared luminol solution increased (oxidized) to a stable value over a few hours. This solution can be continuously used for 1 week when stored at ambient temperature. Typical FIA peak profiles identified the reproducible dose-dependent quenching effect of tannin on 3-aminophthalate fluorescence. Peak heights were then applied to optimize the proposed method.

Effect of Luminol Concentration on Calibration Curve. Although increasing the luminol concentration results in elevated baseline fluorescence and, thus, increased noise, it also increased the sensitivity and dynamic range (**Figure 4**). For improved sensitivity, low background fluorescence and low reagent consumption (0.4 mM luminol) was employed in the following experiments.

Effect of Reagent pH. Effects of reagent pH value were investigated with 0.4 mM luminol in 0.1 M carbonate buffer at varying pH values (**Figure 5**). The background signal decreased slightly with the increase in the reagent pH; consequently, the FIAgram will be immune to easy disruption by transient pH change in the injected sample plug. The region around pH 10 is the most stable and has the highest buffer capacity. The peak height signal was almost unaffected by pH, and occurred at pH > 10. For baseline stability and signal sensitivity, a system pH of 10.0 was adopted.

System Performance. Under optimal conditions (0.4 mM luminol in 0.1 M carbonate buffer, pH 10.0; flow-rate, 0.5 mL/min), the detection limit was 0.001 mg/mL (S/N > 3), and the linear range for tannic acid was 0.005−0.3 mg/mL (r^2 = 0.9896). Relative standard deviations (n = 5) of the signals of 0.1 mg/mL tannic acid were <3%.

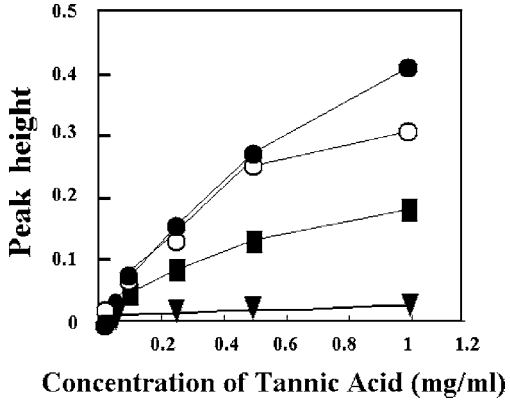

Figure 4. Dynamic ranges for tannic acid with different concentrations of luminol. Luminol concentration: ▼ = 0.04 mM, ■ = 0.2 mM, ○ = 0.4 mM, ● = 1 mM. Flow rate: 0.5 mL/min. Luminol was dissolved in 0.1 M carbonate buffer (pH 10.0). The fluorescence intensity was not calibrated, and the ordinate is scaled as an arbitrary unit.

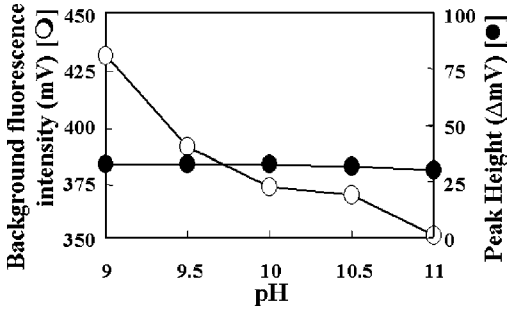

Figure 5. Influence of pH value on background fluorescence (○) and peak height (●). Luminol, 0.4 mM; tannic acid, 0.1 mg/mL; flow rate, 0.5 mL/min. The background fluorescence and the peak height signals were converted into voltage signals (mV) through the same attenuating circuit for intensity comparison.

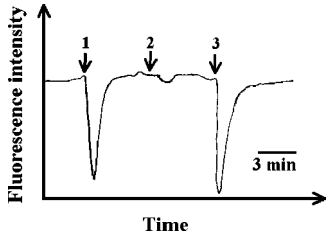

Figure 6. Interference by ascorbic acid. 1, 0.1 mg/mL tannic acid; 2, 1.0 mg/mL ascorbic acid; 3, 0.1 mg/mL tannic acid and 1.0 mg/mL ascorbic acid. Luminol, 0.4 mM; flow rate, 0.5 mL/min. The fluorescence intensity was not calibrated, and the ordinate is scaled as an arbitrary unit.

Interference of Ascorbic Acid. Ascorbate content of fresh tealeaves is typically less than 10% (w/w) of tannin content; however, commercial tea beverages generally add an excess amount of ascorbate to prevent tannin oxidation. Oxidation/reduction-based tannin assays are unsuited to analysis of green tea and commercial beverages. Consequently, a ferrous tartrate method, a complexation-based method, was adopted as the Japanese official method.

To assess the influence of ascorbate on the proposed method, signals of tannic acid (0.1 mg/mL), ascorbate (1.0 mg/mL), and ascorbate (1.0 mg/mL)-spiked tannic acid (0.1 mg/mL) solutions were compared (**Figure 6**). The effect of the ascorbate signal was not significant, even at concentrations 10 times higher than that of tannic acid. Because ascorbate content in ascorbate-adulterated green tea products is lower than tannin content, the contribution of ascorbate signal is negligible. Thus, the proposed

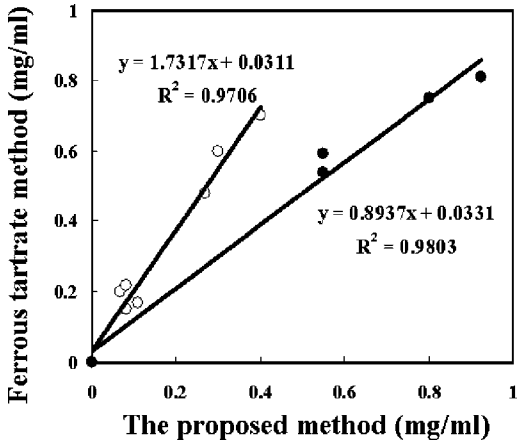

Figure 7. Correlations of data obtained by the ferrous tartrate method and the proposed FIA method. ○, data calculated from the calibration curve obtained with commercial tannic acid; ●, data calculated from the calibration curve obtained with green tea tannin.

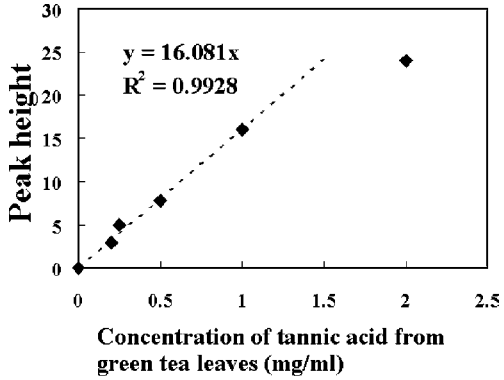

Figure 8. Calibration curve obtained with green tea tannin. The green tea tannin standard was purified as detailed in the Materials and Methods. Luminol, 0.4 mM; flow rate, 0.5 mL/min. The fluorescence intensity was not calibrated, and the ordinate is scaled as an arbitrary unit.

method, like the Japanese official method, is not influenced by the coexisting ascorbate in green tea infusions.

Comparison of Analytical Results for Commercial Green Tea Beverages. Analytical results obtained by the proposed method and the ferrous tartrate method were compared using commercial tannic acid as the standard (**Figure 7**: ○). The correlation is good but has a deviated slope. Because both methods were not affected by ascorbate, a small intersect of the two correlation curves on vertical axis shown in **Figure 7** is expected. The deviated slope is attributable to the difference in method responses to tannic acid standards and green tea tannin and can be resolved by employing a tannin standard with similar tannin compositions.

Tannin can be partially purified by solvent extraction of dried tealeaves as described in the Materials and Methods section. Typically, 0.3 g of tannin extract powder can be obtained from 1.0 g of dried tealeaves. A calibration curve (**Figure 8**) using the tea tannin powder increased the linear range ($r^2 = 0.9928$ for 0.2−1.0 mg/mL), and the correlation of analytical results obtained with the proposed method and the ferrous tartrate method (**Figure 7**: ●) was good with an improved slope.

In conclusion, the fluorometric tannin assay proved effective in directly determining green tea tannin content. Analytical results obtained by the proposed method show high correlations with Japanese official method. Similar to the official method, the assay was not interfered by coexisting reducing compounds, ascorbate, and sample solutions can be analyzed without

pretreatment. However, to improve analytical accuracy, we recommend using tannin standards from representative samples.

Differing from the Japanese official method, the proposed method does not generate precipitating materials, which eased its incorporation into the flow-injection manifold. Moreover, as compared to chemiluminescence detection, fluorometric detection has no signal-decaying phenomenon, and its design, optimization, and automation are more flexible than those of the Japanese official technique. It will be very convenient to accommodate the proposed assay as a routine analytical method and/or eventually to develop a FIA-based analytical machine.

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