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Notes

Selective Determination of Aluminum Bound with Tannin in Tea Infusion

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In this study, an analytical method for indirect measurement of Al bound with tannin in tea infusion was studied. This method utilizes the ability of the tannins to precipitate with protein. Separation conditions were investigated using model solutions. This method is uncomplicated, inexpensive and suitable for real samples. About 34% of the total Al in brew extracted from commercially available teas was bound to condensed and hydrolyzable tannins.

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Introduction

Next to water, tea (*Camellia sinensis*) is the most widely consumed beverage. Tea infusions contain Al in the range of 1 – 6 mg l^{-1} because a tea plant particularly accumulates Al. Al and its salts were considered to be non-absorbable compounds with few chronic or toxic effects. The discovery of a possible correlation between the Al concentration in drinking water and the incidence of Alzheimer's disease has led to a renewed interest in the co-ordination chemistry of Al.[1-6] The bioavailability of Al is strictly linked to the distribution of the element among different chemical species.[3,7]

The studies on speciation of Al in food samples are very limited, although there are many studies on the Al species in water samples. Methods such as ultrafiltration and chromatography have been reported for physical separation of the species. Other speciation methods are based on kinetic discrimination of the species, using fluorometric or spectrometric reagents.[8-10] A few studies have been carried out for the speciation of Al in tea infusions.[10-12] Al species in tea infusion could be categorized into three groups: large organic compounds, small stable organic compounds and free ionic form of Al. Tea contains a large number of compounds that are able to form complexes with Al.[12] It has been assumed that the polyphenols, which account for about 40% of the dry residue of tea infusions, are the most important ligands of Al complexes in tea because they have phenolic hydroxyl groups (such as flavonoid, phenolic acids, coumarin, tannin and lignan) and provide a large number of potential sites for complex formation.[6,13] Polymeric phenols can be divided into two groups; tannin and lignin. Tannins comprise a heterogeneous group of plant polyphenols, all of which are able to combine with skin proteins. The plant tannins have been defined as water-soluble phenolic compounds with molecular weights from 500 to 5000, which perform the usual phenolic reactions and have the ability to precipitate proteins by forming stable cross-linking with their phenolic hydroxyl groups.[14-18]

Al is a metal with very strong "A type" or hard properties, so it strongly prefers oxygen-donor ligands, notably with phosphate, carboxylate and phenolate functional groups. Particularly strong chelate type complexes of Al may be formed with molecules containing oxygen donor functional groups.[19] Studies of spectrophotometric scanning[1,3,20] indicated that Al forms complexes with polyphenols. Nagata *et al*.[21] studied Al forms in intact tea leaves by ^{27}Al-NMR spectroscopy. For young leaves, the single Al signal dominated in the NMR spectrum. This signal agreed with the signal obtained from a model Al-catechins complex. Determination of Al ion bound with tannin is important because both epidemiological experiments and laboratory results have indicated that polyphenols were useful against numerous diseases. Tea inhibits the activity of several enzymes related to tumor ability to scavenge free copper and iron ions, thus preventing oxidative damage. Phenols form highly stable complexes with Al and this process makes Al more biological available.[1-3] If these polyphenols are decomposed in the gut, Al bound with tannin could become more bioavailable.[6] Studies on Al species under stomach conditions before and after gastrointestinal digestion were published.[10] Ultrafiltration studies showed that as much as 86% of the 0.45 μm filterable Al in tea infusions was retained by a 20000 Da molecular-mass cut-off filter and 96% was retained by 10000 and 500 Da molecular-mass cut-off filters.[22] This indicates that the large part of Al in tea infusions exists as large organic complexes. In the study on speciation, size exclusion chromatography was usually used and Al species bound with organic ligands were separated according to molecular size. Most studies on polyphenol-Al complexes were carried out using such model solutions.[1,3] Owen *et al*.[10] used size exclusion chromatography coupled with ICP-MS to study the behaviors of Al in tea infusion before and after simulated gastrointestinal digestion and indicated that about 14% of the Al existed as stable species.

This study involves the formation of protein-tannin complex for indirect determination of Al bound with tannin in tea infusion. For this purpose, the analytical method based on the ability of the polyphenols to precipitate with protein was used. A well-known method for determination of tannin[23,24] was employed for the first time for Al speciation.

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Experimental

Apparatus

Al concentrations were determined with a Philips PU 9100X Model atomic absorption spectrometer. An Al hollow cathode lamp and an optimized nitrous oxide–acetylene flame were used. A Shimadzu 1601PS Model UV-VIS spectrophotometer and a Jenway 3010 Model digital pH meter were used. Glassware and equipment that were Al-free were purchased from Cole Palmer (USA) and were used after the following procedure: soaking in 2 – 4% (v/v) nitric acid overnight and then rinsing with distilled deionized water.

Reagents

All reagents used are of p.a. quality. Merck supra pure acids and deionized water from a Milli-Q system (Millipore, USA) was used for preparing all solutions. Two grams of bovine serum albumin (BSA) (RIA grade fatty acid and globulin free, Sigma, USA) were dissolved in 100 ml of 0.2 M acetate buffer (pH 5.2) containing 0.17 M NaCl. A stock solution of 1000 mg l^{-1} tannic acid, gallic acid or pyrogallol (Merck, Darmstadt, Germany), was prepared by dissolving 1.000 g of each compound in 100 ml of deionized water in a ultrasonic bath, and then filling up to 1 l with deionized water. Standard solutions of Al were freshly prepared from 1000 mg l^{-1} of stock Al solution (Merck). Then, 1% sodium dodecylsulfate (SDS, Merck) and 5% triethanol amin (TEA, Merck) were prepared.

Folin Denis reagent was prepared by dissolving 10.0 mg of sodium tungstate ($Na_2WO_4{\cdot}2H_2O$ Merck) and 2.0 g of phosphomolibdic acid ($H_3PMo_{13}O_{14}{\cdot}H_2O$ Merck) in deionized water, under boiling temperature, then filling up to 100 ml after cooling. Special care should be taken to avoid contamination with organic matter during preparation. The reagents and all stock solutions were stored at 3˚C in polyethylene containers.

Material

Four different commercial types of Turkish black tea were used. Two grams of powdered tea leaves dried at 95˚C for 12 h were placed in a beaker; then 50 ml of hot deionized water was poured over them and the mixtures were heated up to the boiling point. After cooling, the extract was filtered through a piece of Whatman No. 541 filter paper and was filled up to 100 ml with deionized water. The pH values of tea infusions were 4.9 – 5.3.

Procedures

Analysis of total polyphenols in tea infusion. The method reported by the Association of Official Analytical Chemists (AOAC, Official Methods of Analysis 1990, 15th ed., Arlington, Virginia, USA), was used for the determination of the concentrations of polyphenols. Tannic acid was used as the standard of polyphenols in tea infusion. Standard solutions of tannic acid at 2, 4, 6, and 8 mg l^{-1} were prepared by mixing 50, 100, 150 or 200 µl stock solutions of tannic acid with 2 ml of saturated Na_2CO_3 solution and 2 ml of Folin Denis reactive, then filling up to 25 ml with deionized water. After we kept the container standing for 15 min to complete the complex-forming reaction, absorbance at 750 nm was measured. For samples, 0.25 ml tea infusion was used instead of tannic acid. Spectrophotometric measurements were performed according to blank solutions containing all reactives except tea infusion.

Separation of Al ion bound with tannin in tea infusion. For separation of Al bound with tannin in tea infusion, 15 ml of tea infusion was mixed with 6 ml of 20 g l^{-1} BSA solution and this mixture was kept in a water bath at 90˚C for 30 min. After centrifugation at 8000 rpm for 15 min, the concentration of Al in the supernatant was determined by FAAS. Al concentrations in tea infusion were also determined by FAAS.

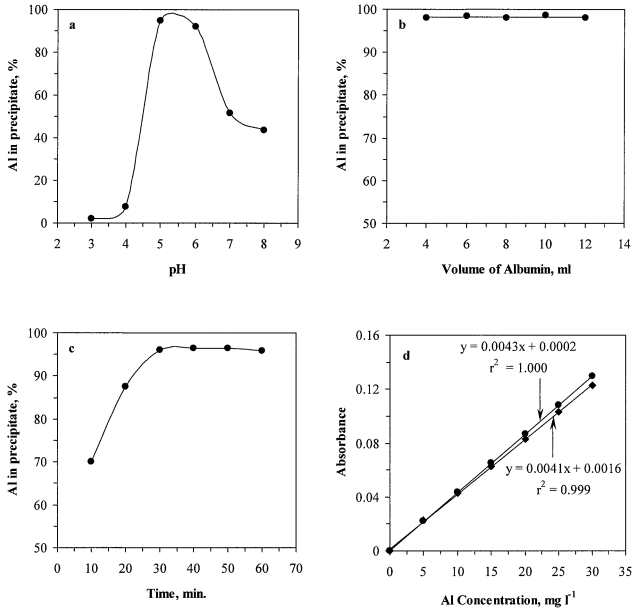

Fig. 1 The effect of (a) pH of the medium (20 mg l^{-1} Al; 250 mg l^{-1} tannic acid; 6 ml of BSA; 90˚C; 30 min), (b) amount of BSA (20 mg l^{-1} Al; 250 mg l^{-1} tannic acid; pH 5.2; 90˚C; 30 min) and (c) precipitation time (20 mg l^{-1} Al; 250 mg l^{-1} tannic acid; 6 ml BSA; pH 5.2; 90˚C) on the recovery of Al in the precipitate. (d) Calibration lines of aqueous Al standard solution (●) and Al solution containing 8 g l^{-1} BSA (◆).

Results and Discussion

Protein precipitation for separation of aluminum bound with tannin

Appropriate standards of polyphenols, pH values of medium, amounts of BSA to be added, and precipitation times were investigated using model solutions. BSA was added to 10 ml of 20 mg l^{-1} Al solution containing 250 mg l^{-1} tannic acid, gallic acid, or pyrogallol at pH 5. The mixtures were left in a water bath at 90˚C with stirring at constant speed for 30 min and then centrifuged at 8000 rpm for 15 min. The Al concentrations in the precipitates were determined after dissolving with 10 ml of 1% SDS, and Al concentrations in the supernatant were also determined. The reagent blank containing all reactives except polyphenol was prepared. All experiments were repeated five times and the concentration of Al was measured three times.

In order to optimize pH conditions of precipitation, we changed the pH values of the model solutions from 3 to 8 with 0.2 M CH_3COOH or 0.1 M NaOH. As seen in Fig. 1(a), the best results were obtained at pH 5. Only 5.5% of Al remained in the supernatant; in other words, 94.5% of Al precipitated with BSA at pH 5. It was observed that Al recovery in the supernatant increases at pH values either lower or higher than pH 5. This result indicated that the optimum pH of the buffer for protein precipitation of tannins was limited to a narrow range, as reported by earlier workers;[18,23] in the present assay the best results were obtained at pH 5. At pH values higher than 5, there was no precipitation, which can be attributed to the

Table 1 Concentrations of Al in the supernatant after precipitation with BSA and in precipitate

Polyphenol	Initial Al conc./mg l^{-1} (A)	Al in the supernatant after precipitation/mg l^{-1} (B)	Difference/ mg l^{-1} (A – B)	Recovery of Al in the supernatant, %	Al in precipitate obtained by dissolving in the detergent solution/mg l^{-1}
Pyrogallol	20.0	20.10 ± 1.85	—	100.50 ± 9.20	—
Gallic acid	20.0	19.47 ± 0.55	—	97.35 ± 2.75	—
Tannic acid	20.0	1.100 ± 0.16	18.90 ± 2.70	5.50 ± 1.30	17.93 ± 4.40

The concentration of polyphenols was 250 mg l^{-1}. Mean ± SD (n = 5).

Table 2 Concentration of total polyphenol expressed as tannic acid, Al in tea and Al bound with tannin

Infusion	Tannic acid/mg l^{-1}	Total Al in tea infusion/mg l^{-1}	Al in precipitate/mg l^{-1}	Al bound with tannin, %
Tea 1	317.62 ± 1.52	11.80 ± 0.78	3.37 ± 0.98	28.56
Tea 2	372.88 ± 1.25	7.90 ± 0.62	3.40 ± 0.47	43.03
Tea 3	355.93 ± 2.51	9.80 ± 0.70	2.52 ± 0.26	25.71
Tea 4	300.00 ± 3.07	8.60 ± 0.67	3.32 ± 0.49	38.60
Mean	336.61 ± 33.61	9.52 ± 1.70	3.15 ± 0.42	34

Mean ± SD (n = 5).

ionization of phenolic groups of polyphenols, making them unavailable for hydrogen bonding with protein.[23,24] Both the pH and ionic strength influenced the precipitation reaction; the suitable condition was 0.2 M acetate buffer solution (pH 5) containing 0.17 M NaCl.

In order to determine the optimum amount of BSA for complete precipitation, we added 4 – 12 ml of BSA solution at pH 5 were added to the model solution. As shown in Fig. 1(b), the recovery rate did not change under the present conditions. Therefore, the volume of BSA solution was fixed at 6 ml.

Different precipitation times (5 – 60 min) were also examined to predict the optimum duration time. As shown in Fig. 1(c), the precipitation time of 30 min was enough for complete precipitation, because the recovery of Al in supernatant did not change with longer time. In the literature, the time for complete formation of tannin protein complex from 15 min to 24 h has been recommended.[23,24] This parameter is thought to vary depending on the nature and quantity of tannin used in each investigation.

The effect of BSA on Al measurement by FAAS

The effect of BSA on Al measurement by FAAS was checked. As seen in Fig. 1(d), there is almost no effect of adding BSA on the slopes of the calibration lines, indicating that measurement of Al by FAAS is not strongly affected by BSA at 8 g l^{-1} or lower. Therefore, calibration curves obtained with aqueous Al standard solutions were used in the present study.

Choice of appropriate polyphenol standard

BSA was added into the model solutions to choose an appropriate polyphenol standard. After adding BSA, a jelly white precipitate formed only in the model solution containing Al bound with tannin, whereas no precipitation was observed in model solutions containing Al bound with pyrogallol or Al bound with gallic acid. Tannic acid, therefore, was chosen as a suitable polyphenol standard for precipitation with BSA. Differences in precipitation properties of tannin, pyrogallol and gallic acid indicated that the monomeric flavan-3-ols (catechin) and gallic acid, polyphenol with molecular weights less than 500, did not precipitate protein. They cannot be classified as plant tannins.[25] A precipitation of the protein occurs only in the case that several peptide chains are bound to the same phenol molecule.

Al recoveries in the supernatant after centrifuging the BSA added solution are given in Table 1. 94.5% of Al bound with tannin was precipitated with BSA. Al concentration in precipitate was also determined experimentally by dissolving the Al bound with tannin-protein (BSA) complex in 10 ml of detergent solution containing 1% SDS and 5% (v/v) TEA (Table 1). When the Al concentration in the supernatant is subtracted from the initial Al concentration, the fraction of Al bound with tannin precipitated by BSA can be calculated. The results obtained by calculation were found to be similar to those obtained from the precipitate. Since repeatability of the results obtained by dissolving precipitate was inferior, and the measurement of Al in the supernatant was more reliable, practical and less time consuming, Al concentrations in precipitate were calculated from Al concentrations in the supernatant in other experiments. It has been reported that the co-precipitation of tannin with protein occurs in a two-stage mechanism, including initial complexation and subsequent precipitation of the complexes. In the initial complexation, the number of galloyl groups in a galloyglucose molecule is important (penta > tetra > tri > di > mono); at least three galloyl groups are necessary for effective complexation.[26]

Analysis of total polyphenols in tea infusion

The total polyphenol concentration was determined using tannic acid as standard. The Folin Denis colorimetric method is the official method in the U.S., and is perhaps the most widely used method for determination of total phenols and of tannins in a wide range of plant products and beverages. Catechin and tannic acid are often used as the standard of polyphenols. Their absorption characteristics greatly differ from each other. Due to the absence of linearity of the catechin standard curve using the Folin Denis method, it vastly overestimates the tannin content.[18] The average concentration of total polyphenols indicated as tannic acid in tea infusions from different origin was found as 336.61 ± 33.61 mg l^{-1}. Table 2 gives concentration of total polyphenols obtained by Folin Denis method for each tea infusion.

Determination of total Al ions and of Al ions bound with tannin in tea infusion

Al in tea infusion and that in precipitate are listed in Table 2. A considerable number of studies have reported the total concentration of Al in tea infusion. The average concentration of Al of four different commercial black tea samples from different localities in Turkey was 9.52 ± 1.70 mg l^{-1}. The content of Al in these samples is higher than that of any other country's tea.[6] Variation of Al levels in tea leaves may reflect different soil conditions and/or contamination of Al during the fermentation process of the black teas. The result indicated that about 30% of Al in dry tea leaves was transfered into tea infusion. This ratio agrees with that in previous reports.[6,27] Erdemoğlu *et al*.[27] used two different ion exchange resins for speciation of Al in Turkish black teas. XAD-7 resin was used for separation of organic species of Al; 28 – 33% of the Al in tea infusion was sorbed to the non-ionic XAD-7 resin. 10 – 19% of Al bound to the Chelex 100 resin may consist of cations such as Al^{3+} and its hydrolysis products, Al-fluoride and cationic organic complexes.

Table 2 indicated that about 34% of Al in tea infusion was bound with condensed or hydrolyzable tannins that could be precipitated with BSA. This result is in good agreement with the ratio of Al bound with organic compounds, 28 – 33%, reported in the literature.[27]

The possible species of Al remained in the supernatant after BSA precipitation may include: Al bound with other polyphenols but not precipitated with BSA, Al bound to organic molecules other than polyphenols, free Al^{3+} ions, or inorganic Al complexes such as AlF^{2+}.

Conclusions

Al bound with tannins was found in tea infusion. Among many polyphenols, only polyphenols with tannin structure can be precipitated with BSA and they were easily separated. This method was found to be very simple, cheap, less time-consuming and useful for measuring Al bound with tannin in tea infusion.

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