Effect of Addition of Aluminum Salts on the Quality of Black Tea

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Addition of an appropriate Al salt increases redness and brightness of extracts of black tea and also improves the taste. An extensive study was carried out to determine which tea constituents reacted with Al to improve tea color. Extraction with solvents indicated that the theaflavins reacted with Al salt to form red complexes. Separations by Sephadex LH-20 chromatography and HPLC showed that at least two other tea polyphenols formed bright, deep yellow complexes with Al salt. Several other tea components also reacted slightly with Al salts to form yellow complexes. However, stoichiometrically most of the Al in tea is in the uncolored forms. Al ions appeared to be unique among common cations in being capable of enhancing tea color and flavor.

Among the characteristics of a good cup of black tea are a red or rosy color (especially when milk is added) and briskness without bitterness. The chemical entities in tea that are generally believed to cause variations in color and bitterness are chiefly the theaflavins and thearubigins. The quantities of each, and the ratio of their quantities, are said to determine both the color characteristics of the tea brew and its taste (Roberts and Smith, 1963; Sanderson, 1972). For example, a tea with a high level of theaflavins and a low level of thearubigins would tend to give a brew with a yellow-orange color and a high degree of briskness. Conversely, a tea with a low level of theaflavins and a high level of thearubigins would be expected to give a brew with a brown color and little briskness (a soft tea). A tea with an optimum level of each of these chemical groups may give a brew with a rosy color and appropriate briskness.

It is generally accepted that ionic impurities found in tea-brewing water (such as sodium, calcium, iron, and chlorine) adversely affect both the taste and color of the brew, and distilled water is therefore said to be the best brewing water (Roberts and Smith, 1963; Basu and Ullah, 1974).

Recently, however, the beneficial effects of aluminum salts on tea quality were discovered by Edmonds and Gudnason (1979). These investigators found that treating tea and tea compositions with an aluminum salt increased their redness and brightness and improved the taste quality. The improvements took place whether the aluminum was added before, during, or after tea fermentation.

Interestingly, the aluminum content of plucked tea leaves ranges from 200 to 1000 ppm (dry weight basis) compared with 50 to 100 ppm in other plant species. Variations in the amount of aluminum in plucked tea leaves may or may not affect qualities of black tea infusions significantly. The effect on quality depends on how much aluminum is actually extracted and the amount of theaflavin and thearubigin in the extract. Preliminary work on the aluminum tea reactions showed that mainly theaflavins were involved in the formation of red compounds. Aluminum–polyphenol complex formation has been reported before by Geissman (1962). More extensive studies on the effects of Al addition on tea constituents are reported here.

MATERIALS AND METHODS

Reagent and chromatography grades of chemicals were purchased for use from various U.S. suppliers. Since pH variations may affect tea color, unless otherwise stated, we

used controls with pH adjusted to those of the treated samples for valid comparison.

Theaflavin Analysis. The traditional method (Roberts and Smith, 1961, 1963) was modified by extracting with ethyl acetate instead of isobutyl methyl ketone. The-aflavins were expressed as percentages of total soluble solids.

Sephadex LH-20 Chromatography. A glass column (5-cm i.d., Laboratory Data Control Model LC-2-13) was packed with Sephadex LH-20 (Pharmacia, Superfine powder) in 60% acetone to a height of 18 cm. The column was eluted with distilled deionized water until all acetone was removed. The tea sample in water was then applied onto the column. A stepwise elution was carried out with water and 30–60% aqueous acetone solutions at a flow rate of about 5 mL/min. Fractions of 20 mL were collected. The column effluents were monitored at 380 nm.

High-Pressure Liquid Chromatography (HPLC). The HPLC method of Hoefler and Coggon (1976) was used to determine the theaflavins and other constituents of tea.

Atomic Absorption Analysis of Aluminum. The samples were ashed (Association of Official Analytical Chemists, 1975), and the ash was suspended in hot H_2O adjusted to pH 4 with HCl. Aluminum was determined in appropriate dilutions of the filtered solutions along with a series of standards on a Perkin-Elmer 403 atomic absorption spectrophotometer, with an HGA-2100 controller (drying, 110 °C for 60 s; charring, 525 °C for 90 s; atomizing, 2500 °C for 8 s) and a graphite cell. The wavelength was set at 309 nm.

RESULTS AND DISCUSSION

Effects of Al Salts on Whole Tea Samples. All infusions of commercial black teas tested gave a redder and brighter liquor color upon addition of an Al salt. The intensity of the color produced varied with the tea used and with the amount of Al salt added. The color reaction was noticeable when Al was added at a level as low as 50 mg/kg of tea solids. The redness, expressed as absorbance at 525 nm (Y), increased linearly with the amount of added Al, in micrograms Al-per gram of tea solids (X). Reconstituted dried extracts of black teas responded in a similar fashion. For example, a 0.43% hot water soluble instant tea gave the following linear relationship for $X = 0$–10 000: $Y = 1.033 + 0.00014X$ (correlation coefficient = 0.999). However, hot water infusions of green tea leaves or reconstituted instant green teas did not redden upon addition of Al salts. Furthermore, tea extracts from dried steamed green tea leaves also failed to give the red reactions. In addition to enhancing the color quality of the black tea extract, the taste was also improved appreciably

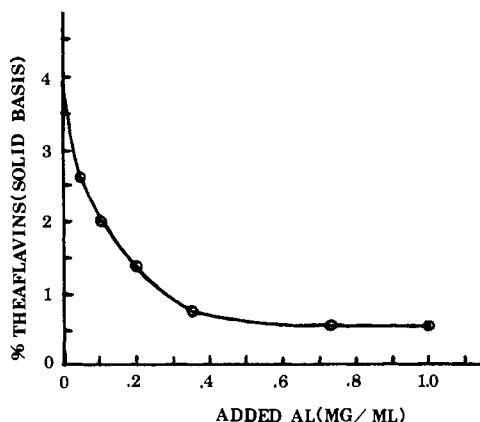Corporate Research and Development Department, The Coca-Cola Company, Atlanta, Georgia 30301.

Figure 1. Effect of $AlCl_3$ addition on theaflavin contents of a hot water soluble instant tea solution (0.43% total solids) as determined by the ethyl acetate extraction method.

when aluminum salts were added, in that the tea was smoother.

Effects of Kinds of Al Salt Used. Aluminum sulfate, aluminum chloride, aluminum potassium sulfate, and aluminum nitrate were found to give the same color reactions in the tea. Aluminum hydroxide did not react to give color. However, red Al–tea products were formed on the insoluble aluminum hydroxide freshly prepared from neutralization of either acid [$AlCl_3$, $Al_2(SO_4)_3$] or alkaline ($NaAlO_2$) Al salts. Addition of aluminum citrate to tea resulted in gradual color reaction. The color intensity obtained with aluminum citrate was much less than with other soluble Al salts. This may be related to the different degrees of ionization of the Al salts.

Effect of Al on Theaflavins and Thearubigins. As shown in Figure 1, the theaflavin content of a tea extract appears to decrease as increasing amounts of Al salt are added. The red Al–tea products were not extractable by ethyl acetate, except when aluminum citrate was used to form red products in tea, presumably due to an Al-theaflavin–citrate complex. When an Al salt was added to a thearubigin fraction, no red color was formed.

Effect of Citrate, Fluoride, and EDTA. Addition of EDTA, fluoride, or citrate to tea containing added aluminum resulted in a significant reduction of the color reactions due to Al. However, the three chelating agents reacted differently.

(*a*) *Effect of Citrate.* Although addition of aluminum citrate to tea produces a red-colored reaction as mentioned above, addition of a pH 5.0 sodium citrate solution to tea containing added aluminum reduced absorbance of the tea at 525 nm. As the citrate concentration was increased from 0 to approximately 0.01 M, absorbance was reduced by 80%. Higher citrate concentrations caused very little further decrease in the 525-nm absorbance. The citrate reaction was almost complete in approximately 1 h.

(*b*) *Effect of Fluoride.* Addition of potassium fluoride to tea containing added aluminum reduced absorbance at 525 nm, as the concentration of fluoride was increased from 0 to 0.05 M. Beyond 0.05 M, however, the absorbance increased with increased fluoride concentration, due to formation of dark brown colors from reactions between fluoride and tea. The color-reducing effect of fluoride on Al-induced tea color was much less than that of citrate. The formation of the stable aluminum hexafluoride ions is probably responsible for the color-reducing effect.

(*c*) *Effect of EDTA.* An effect similar to that of citrate was observed with 0–0.01 M Na_2EDTA. Addition of more than 0.01 M caused the 525-nm absorbance to decrease but at a decreasing rate. However, at EDTA concentration of
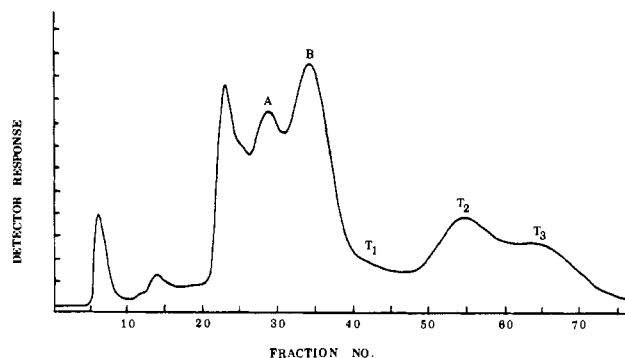

Figure 2. Sephadex LH-20 chromatography of a hot water soluble instant tea (0.5 g). The ordinate represents absorption at 380 nm at 2.0 aufs. The column was first eluted with H_2O for seven (20 mL) fractions and then with 50% acetone in H_2O for the rest of the run. Addition of Al salt to fractions of the theaflavin peaks T_1, T_2, and T_3 and those of peaks A and B yielded red and yellow Al complexes, respectively.

0.08 M and above, the 525-nm absorbance was reduced to even below that of the original tea infusion itself (without Al added). This is probably due to disruption of the naturally occurring aluminum–theaflavin complexes in tea.

Effect of Al on Chromatographic Fractions of Tea. Sequential elution of tea on Sephadex LH-20 with water and aqueous acetone solutions (Figure 2) yielded, in order, high, intermediate, and low molecular weight materials which did not interact with Sephadex LH-20 (fraction no. 5–25). Most colored tea components, however, interacted with the column material. Most of them could not be eluted with water, but they were elutable with aqueous acetone (peaks A, B, T_1, T_2, and T_3). The last three peaks (T_1, T_2, and T_3) are the theaflavins, which have characteristic maxima at 380 and 460 nm. All of the early peaks showed only decreasing optical absorption from the near-UV region to longer wavelengths without distinct 380- and 460-nm absorption maxima.

Addition of Al salt to the chromatographic components generally caused bathochromic shifts. Thus, the 380- and 460-nm maxima of the theaflavins were shifted to about 400 and 510 nm, respectively. The color of fractions from the two peaks eluted immediately before the theaflavins (peaks A and B, Figure 2) changed from light yellow to deep, bright yellow, with an accompanying absorption shift from the UV region to the near-UV region of the visible spectrum. The absorption curves of the other peak fractions also shifted slightly. The reddish Al–tea color reactions were mainly due to the 510-nm absorption maximum shift. It is thought that the bright, deep yellow Al products formed with peaks A and B may enhance the brightness of a tea liquor.

HPLC patterns of tea itself and tea with an Al salt added (Figure 3) clearly showed the effects of Al on the various HPLC peaks. The heights of all the theaflavin peaks and most of the other peaks were reduced upon addition of Al to the tea. An increase in the early peak may be due to some polar Al–tea reaction products.

A reconstituted dried extract of black tea with up to 8000 μg of Al (as $AlCl_3$)/g of tea solids added was chromatographed on Sephadex LH-20 to compare the elutions of the tea with and without added Al salts. In addition to the regular tea components, Al-treated tea gave a pink high molecular weight components, a pinkish orange component eluted at the early stage of aqueous acetone elution, and an aqueous acetone eluted component that precipitated after elution. Furthermore, whereas all the usual tea components were eluted by sequential elution with water and aqueous acetone solutions, the tea sample treated with
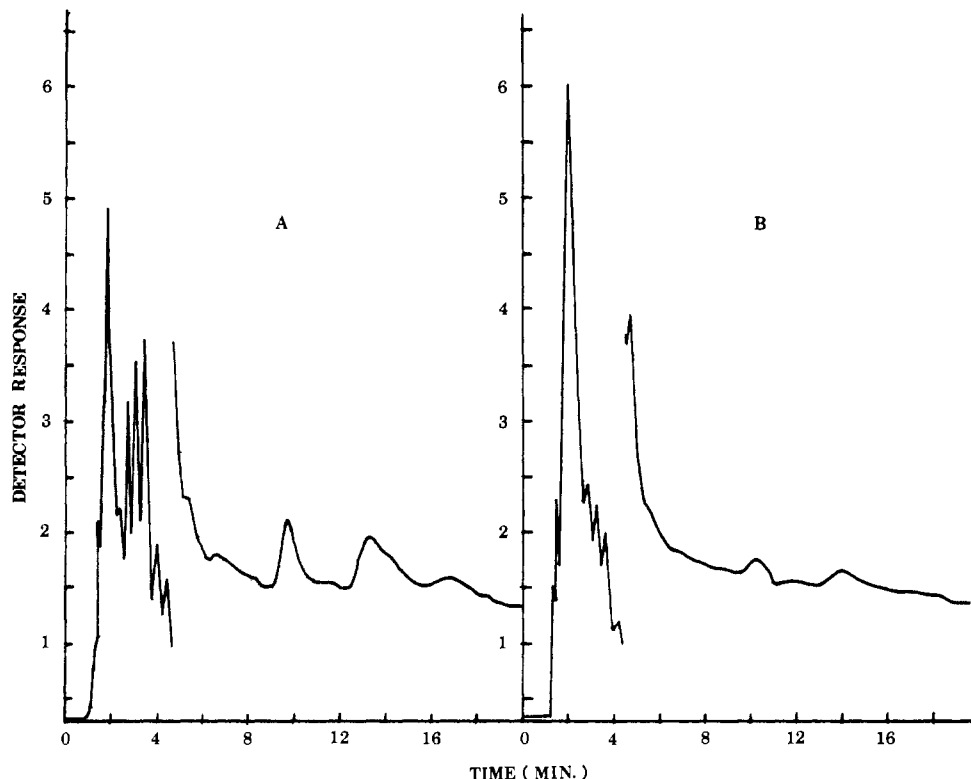
Figure 3. Effect of Al salt addition on HPLC patterns of a black tea infusion. Chromatograms A and B were obtained from a 2-mL infusion with 0.5 mL of H_2O or 0.5 mL of 1% $Al_2(SO_4)_3 \cdot 16H_2O$ added, respectively. Conditions: Waters μBondpak C_{18} column; mobile phase, acetic acid–acetone–H_2O (1:60:139); flow rate, 2.0 mL/min; room temperature; pressure, 3000 psi; detector, 365 nm at 0.2 aufs for the first 5 min and 0.05 aufs afterward to show the theaflavin peaks.

$AlCl_3$ contained pink material that was retained on the top of the column, after elution with water and 0–60% acetone. The strong affinity of the reddish Al–tea reaction products to the Sephadex may be attributable to the dual ionic–covalent character of Al and its ease of coordination with hydroxyl groups present in the Sephadex resin.

Efforts were made to elute and characterize the Sephadex-bound Al–tea reaction products. Following the chromatographic run of the tea sample with added $AlCl_3$ as above and a water elution to remove acetone from the column, a pH 6.0, 0.5 M sodium citrate solution was used to strip the Al from the Al–tea–Sephadex reaction products, since aluminum citrate was a more stable compound. The Al-stripped tea solids remaining in the column were then eluted by sequential water and aqueous acetone as before. Spectrophotometric characterization of the column fractions indicated that the tea components that had been bound as Al–tea reaction products included theaflavin and nontheaflavin components. As expected, the theaflavins formed reddish reaction products with Al, whereas the two peaks eluted immediately before the theaflavins (Figure 2, peaks A and B) formed yellow products. Neither type of Al products was elutable from the Sephadex column with H_2O or aqueous acetone. Total solid determinations of the freeze-dried fractions revealed that only a very small proportion of the tea components had reacted to form the Al–tea reaction products. Most of the tea color was eluted by H_2O and aqueous acetone.

Distribution of Al in Tea among Sephadex LH-20 Fractions. A reconstituted dried extract of black tea containing 200 μg of added Al/g of tea solids was eluted from Sephadex LH-20 consecutively with water, 30% acetone, and 45% acetone. Determination of Al by atomic absorption showed that 93.9% of the total Al in tea was eluted by H_2O. The Al remaining on top of the column after aqueous acetone elutions accounted for approxi-

mately 2.7%. The Al eluted by aqueous acetone was only 3.4%. It appeared that most of the Al in tea was bound to noncolored or weakly colored tea components and that only a few percent was complexed with the main tea polyphenols.

Effects of Addition of Various Inorganic Salts on Color of Theaflavin Solutions. Theaflavin fractions from Sephadex LH-20 chromatography of whole tea were used to study these effects. It was found that $AlCl_3$ caused the greatest color effect, changing the yellow-orange color into a pinkish red color. $FeCl_3$ gave a gray-purplish orange color. NaCl, $MnSO_4$, and $CaCl_2$ did not affect color of the theaflavins in solution. Slight enhancement of the yellow color was observed with $ZnCl_2$ and $MgCl_2$. $SnCl_2$ and $CuSO_4$ gave a slight increase in orange color. $SnCl_4$ precipitated the theaflavins, but the color of the suspension was similar to that of the theaflavin solution.

We conclude that the aluminum ion is unique among the inorganic cations in being capable of increasing and improving color in teas that contain significant levels of the theaflavins and also improving the taste. Although Al is naturally abundant in tea, its role in color formation and its effect on flavor had not been noted until the discovery of Edmonds and Gudnason (1979). Previous work in this laboratory indicated that only a fraction of the naturally occurring Al is extractable from tea leaves. As shown earilier, of the extracted Al, less than 3% existed as theaflavin complexes. This explains why addition of very small amounts of an Al salt causes a significant color improvement, even in extracts from tea leaves with high total Al content.

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Synthesis of Haptens and Potential Radioligands and Development of Antibodies to Insect Growth Regulators Diflubenzuron and BAY SIR 8514

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A variety of synthetic approaches were undertaken, leading to potential haptens and radioligands for the benzoylphenylurea insect growth regulators diflubenzuron and BAY SIR 8514. One successful approach involved derivatization of the aniline nitrogen by ethyl 4-bromobutyrate followed by reaction with an appropriate isocyanate and cleavage of the ethyl ester to yield a free carboxypropyl "handle". Useful haptens were also synthesized by using a 3'-phenolic metabolite of diflubenzuron as well as acetate and amine functionalities in the 4' position while the N-sulfenyl bond proved too unstable for use as an antigen. With the exception of the sulfenylated derivatives, the haptens lacked significant biological activity on three insect species. Following protein coupling by the active ester or water-soluble diimide method, antibodies were raised to two diflubenzuron haptens in each of seven rabbits immunized as demonstrated by radioimmunoassay using [¹⁴C]diflubenzuron, Ouchterlony gel diffusion, and immunoelectrophoresis.

The insect growth regulators (IGRs) diflubenzuron [Dimilin, TH-6040, N-[[(4-chlorophenyl)amino]-carbonyl]-2,6-difluorobenzamide, 1] and BAY SIR 8514 [N-[[[4-(trifluoromethoxy)phenyl]amino]carbonyl]-2-chlorobenzamide, 18] are promising insect control agents. Since these compounds and structurally related IGRs are either in use or projected for commercial use, information regarding their environmental fate is exceedingly important. Numerous studies have been carried out on the persistence and routes of degradation of benzoylphenylurea IGRs under a variety of conditions [see Maas et al. (1980) and Hammock and Quistad (1981) for references]. The classical methods of diflubenzuron analysis by GLC or HPLC are of adequate sensitivity and reproducibility; however, these methods are very expensive and tedious (Corley et al., 1974; Oehler and Holman, 1975; Lawrence and Sundaram, 1976; Maini and Deseo, 1976; Schaefer and Dupras, 1977; Worobey and Webster, 1977, 1978; DeMilo et al., 1978; DiPrima et al., 1978; Rabenort et al., 1978; Monem and Mumma, 1981). So that a large number of samples can be inexpensively analyzed within a short period of time, improved analytical methods are needed. As shown by previous reports on the development of pesticide-specific antibodies, immunochemical procedures offer some advantages over GLC- and HPLC-based methods (Hammock and Mumma, 1980). Therefore a study was undertaken to develop simple immunoassays for the benzoylphenylurea IGRs. This report illustrates the synthesis of diflubenzuron and BAY SIR 8514 protein conjugates and their use in the induction of IGR-specific antibodies.

MATERIALS AND METHODS

Analytical TLC was performed on silica gel F_{254} plates (EM Laboratories), while preparative TLC was done on 2000-μm silica gel plates with fluorescent indicators (Analtech). Compounds were detected by their quenching of gel fluorescence when viewed under shortwave ultraviolet (UV) light (254 nm). TLC systems found useful for this study include toluene–propanol (10:1), toluene–propanol–acetic acid (10:1:0.1), toluene–propanol–NH₄OH (10:1:0.1), hexane–ethyl acetate (3:2), and hexane–ether (5:1). Each stable intermediate and final synthetic product showed only a single spot on TLC in a minimum of three solvent systems including at least one acidic and one basic system. Proton magnetic resonance (¹H NMR) spectra were obtained on a Varian EM-390 in a dilute solution of the indicated solvent with or without deuterium oxide and always by using tetramethylsilane as the internal standard. Infrared (IR) spectra were run as thin films of the respective compound on silver chloride plates or in Nujol with Beckman Model 4240 or Perkin-Elmer Model 700-A spectrophotometers. All reactions were carried out under N₂ in subdued light using dry, freshly distilled solvents. Water was doubly distilled except in the case of saturated brine. An overview of synthetic procedures is shown in Figure 1.

Synthesis. *2,6-Difluorobenzoyl Isocyanate.* To a magnetically stirred suspension of 2,6-difluorobenzamide (98%, 0.075 mol) in 65 mL of dry 1,2-dichloroethane was added freshly distilled oxalyl chloride (0.097 mol). The mixture was then refluxed for 24 h and the solvent distilled off at reduced pressure and the remaining syrup vacuum distilled to give 11.25 g of clear oil in 90% yield: bp 62–63 °C at 0.4 mmHg (bath temperature 90–95 °C); NMR (CCl₄) δ 7.00 (t, 2) and 7.50 (m, 1) and the absence of amine proton signals; IR (neat, AgCl) 2252 cm⁻¹ (s) and the absence of N–H stretch band. 2-Chlorobenzoyl isocyanate was prepared in a similar manner as earlier described by Wellinga et al. (1973a) for 2,6-dichlorobenzoyl isocyanate as shown in Figure 1.

N-[(Ethoxycarbonyl)methyl]-4-chloroaniline (B, Figure 2). A suspension of 4-chloroaniline (0.02 mol), ethyl bromoacetate (0.02 mol), and sodium acetate (0.02 mol) in 5 mL of ethanol was heated to reflux for 8 h. The reaction mixture was poured into 150 mL of water and extracted with ether (2 × 50 mL). The ether layer was

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