Analysis of Tea Polyphenols (44377A)

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Abstract. **Tea is the most highly consumed beverage in the world, other than water. However, unlike water, tea contains substantial amounts of polyphenols that have unique biological activities and may be responsible for many of the health benefits of tea. As a result, it is essential to be able to measure the various tea-associated polyphenols. Total polyphenol content is currently measured by using methodology based on reducing activity. Several HPLC systems with detectors that, collectively, have wide ranges in sensitivity have been developed for analysis of individual flavonoids in tea and biological samples, and for theaflavins in tea. Catechins also have been measured in plasma by solid phase extraction, addition of a chromophore, and colorimetric quantification. Except for theaflavins in tea, routine and robust methods for the measurement of polyphenol condensation products (dimers and thearubigens) in tea and biological samples have not been developed. Although *in vitro* and animal studies suggest substantial metabolism of flavonoids in the gastrointestinal tract, only a single HPLC procedure has been assembled for monitoring the metabolic products of quercetin in urine of human subjects.** [P.S.E.B.M. 1999 Vol 220]

Tea is the most highly consumed beverage in the world, other than water. However unlike water, tea contains many organic constituents, some of which appear to have medicinal and health benefits that were known to early Chinese civilizations. In addition to protein and carbohydrates, tea contains substantial amounts of polyphenols (1). It is this broad class of compounds with its unique biological activities that may be responsible for many of the health effects of tea and is currently undergoing intensive scientific investigation.

The polyphenols in green tea are predominantly members of three subclasses: the flavanols, the flavones, and the flavonols. Four major flavanols or catechins (flavan-3-ols), (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, and (-)-epigallocatechin gallate, constitute about one-third of the dry weight of green tea (1). Quercetin, kaempferol, myricetin, and their glycosides (flavonols) as well as apigenin glycosides (flavone) are also present but at much lower concentrations. Whereas green tea undergoes little oxidation, the manufacture of black tea, which constitutes about 80% of the tea production in the world, is primarily an oxidation process of tea leaves catalyzed by polyphenol oxidase (1, 2). As a result, many oxidation products of polyphenols, primarily catechins, are present in black tea. The dimeric forms of these products have been identified as theaflavins, theaflagallins, theasinensins and theacitrins (see Ref. 1 for structures). In addition, thearubigens are formed that have been characterized as high molecular weight (1–40 kDa), heterogeneous polyphenolic condensation products thought to be responsible for the dark color of black tea and that are only beginning to be characterized (1). These oxidation products present a substantial challenge to analytical and natural product chemists.

Measurement of Polyphenols in Tea

The measurement of polyphenols in teas has employed a variety of analytical techniques. Estimation of the "total" polyphenolic content of tea has relied on colorimetric procedures such as the Folin-Denis or Prussian Blue assays that are based on "reducing activity" or redox reactions mostly specific to phenolic groups (1). In spite of advances in instrumental techniques, these nonspecific methods are still useful for estimating the amounts of noncharacterized polyphenols in tea and other foods. Another indirect approach to the estimation of the polyphenol content of tea is the measurement of radical scavenging activity. This approach is similar to the "total" polyphenol assay because radical scavenging activity has been linked to redox potentials (1, 3).

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Several techniques have been developed to measure radical scavenging activity; however, the method that has been used to generate most of the data for tea and tea components is the TEAC assay. This method is based on the ability of a molecule(s) or tea preparation to scavenge stable free radicals of ABTS in an aqueous phase (4). The results are compared to the activity of Trolox, a water soluble form of vitamin E, and are expressed as Trolox equivalent antioxidant activities, hence the acronym TEAC. Vitamins C and E have similar scavenging activities as Trolox whereas all of the polyphenols isolated from tea and tested had from 1.5 to about 6 times the activity of the reference compound (4). In particular, quercetin and all of the catechin gallate derivatives (three adjacent hydroxyl groups on a benzene ring) had TEAC values that ranged from 3.8 to 6.2 (4). Both green and black tea, at a concentration of 1 mg/ml, had TEAC values at the lower end of this range.

Several chromatographic techniques have been employed to separate, characterize, and quantify individual polyphenols in teas (5). For routine analysis, high-performance liquid chromatography (HPLC) equipped with a reversed-phase column (RP-HPLC) and an ultraviolet-visible (UV-Vis) detector has become the 'work horse' instrumental method. An example of such a system that employed a linear gradient of either acetonitrile-acetate buffer or methanol-acetate buffer as the mobile phase to quantify the four predominant catechins and caffeine in tea infusions was recently described (6). Similarly, Hertog *et al.* (7) measured the levels of flavonols (quercetin, kaempferol, myricetin) and flavones (apigenin, luteolin) as aglycones in tea infusions with an RP-HPLC system that used either an acetonitrile-phosphate buffer or a methanol-phosphate buffer as the mobile phase. Several other RP-HPLC systems have been developed for the measurement of flavonoids in both green and black tea preparations and were recently reviewed (Merken H, Beecher GR, unpublished data; see also Ref. 5). Recently, Horie and Kohata suggested capillary electrophoresis as a rapid technique for the estimation of those components of green tea that contribute to tea quality (8). Although accuracy and precision data were not presented in this report, the primary advantage of capillary electrophoresis is the relatively short analysis time (about 10 min compared to 30 min for HPLC).

Recent advances in mass spectrometry instrumentation have permitted the characterization of polar molecules, such as polyphenols. Direct analysis of infusions of green tea with electrospray ionization mass spectrometry operated in the negative ionization mode met with limited success in terms of the identification of prominent polyphenols and other minor components (9). Miketova *et al.* evaluated several ionization procedures for mass spectrometry of the prominent catechins found in tea and concluded that the best results were obtained when individual compounds were separated by HPLC prior to mass spectrometric analysis (10). In addition, these investigators found that electrospray ionization mass spectrometry operated in the negative ionization mode while coupled to an HPLC system (LC/ESIMS) gave the most useful qualitative results and the most accurate analytical data.

Accurate analytical systems for the measurement of dimers and polymers of polyphenols in teas and their infusions are only beginning to be developed. Traditionally theaflavins and thearubigens have been quantified by spectrophotometric techniques using specific wavelengths (5). Because of the nonspecificity of this method for the theaflavins, the Flavognost reagent (2-aminoethyl diphenylborate), which forms a green complex with *cis*-1,2-dihydroxy benzoyl groups, was introduced (5). Reeves *et al.* discussed several short-comings of the Flavognost assay that might account for the high intra- and inter-laboratory variation (11). The development of RP-HPLC methods for the separation and quantification of the four primary theaflavins in black tea revealed that the Flavognost procedure greatly overestimated theaflavin content (5). As part of an extensive investigation of phenolic compounds of black tea liquor, Bailey *et al.* developed an extensive RP-HPLC system that employed a linear gradient and a photodiode array detector and that separated and quantified several phenolic acids, catechins, flavonols, and theaflavins (12). Routine separation and analytical procedures have not been developed for the other polyphenol dimers commonly found in black tea.

Thearubigen values have routinely been calculated for black tea from total polyphenol values by the subtraction of estimates for polyphenols such as catechins and theaflavins. Balentine *et al.,* have suggested improvements in these calculations by first basing total polyphenol analysis on a defined polyphenolic mixture from tea rather than on gallic acid and, second, subtracting values for monomeric and dimeric polyphenols as well as gallic acid based on HPLC analysis (1). Bailey and colleagues investigated the structural characteristics of the thearubigen fraction of black tea (12, 13, 14). Although these investigators employed several HPLC and other column chromatography systems as part of their studies, a single procedure did not emerge for the quantification of this complex group of anthocyanidin-like compounds.

Measurement of Polyphenols and Their Metabolites in Biological Samples

Intact, simple polyphenols (flavonoids) when given as a beverage or part of a meal are absorbed to a very limited extent and have a relatively short half-life in plasma of human subjects (15, 16). As a result, analytical systems to detect them in biological samples must have greater sensitivity than those procedures used for measurement in tea or other beverages and foods. Several methods have been employed that quantify total plasma polyphenols. These include total reducing activity as determined by Folin-Ciocalteu reagent (17) and reduction of iron followed by reaction with 1,10-phenanthroline to form a colored com-

plex (18). Both of these methods require background correction because plasma proteins and other reducing substances in plasma also react positively with these reagents. To increase specificity of detection, at least some purification is often required. Kivits *et al.* developed a rapid and sensitive method for the measurement of catechin in plasma by first extracting them with a solid phase aluminum oxide column followed by formation of a colored complex with dimethylamino-cinnamaldehyde, which was quantified colorimetrically (19).

High-performance liquid chromatography (HPLC) is the preferred method for the analysis of individual polyphenols in biological samples. However, ultraviolet-visible detection lacks sufficient sensitivity to be applicable. Most polyphenols can be determined by chemiluminescence detection following reaction with hydrogen peroxide and acetaldehyde. Nakagawa and Miyazawa coupled this method of detection to HPLC separation and measured plasma EGCG levels at less than 1 ng/ml after administration of a single dose to human subjects (20). Most polyphenols also have weak native fluorescence that has been used to measure catechin in rabbit plasma after HPLC separation (21). The fluorescent response of flavonols can be greatly increased by complexing them with a variety of metal ions (22). Based on this concept, several investigators have developed very sensitive post-HPLC detection systems employing aluminum as the metal chelate (23, 24). It is pertinent to note that only those flavonols that contain a free 3-hydroxyl and 4-keto oxygen formed fluorescent complexes with aluminum. Perhaps the most significant recent instrumental advancement in the post-HPLC detection of polyphenols has been the development of the coulometric electrochemical detection system (25). Lee *et al.* developed an elegant method using HPLC with this detection system to monitor plasma and urine levels of catechins after tea had been administered to human subjects (26). This group and others have employed these types of systems to investigate the metabolism of tea polyphenols in humans and laboratory animals (12, 27, 28, Warden *et al.*, unpublished observations). Most polyphenols circulate in the body and are excreted as conjugates of glycosides and sulfate, and as a result, are usually treated with appropriate enzymes to form aglycones during sample processing prior to HPLC separation (26). An exception is when the metabolism of specific polyphenol conjugates is under investigation (15).

To date, research efforts have focused on the absorption and metabolism of the simple polyphenols (flavonoids) found in tea. As a result, sensitive analytical systems have not been developed for the measurement of individual polyphenol dimers, thearubigens, or their metabolites in biological systems. Several investigators have followed the increased levels of "total polyphenols" based on total reducing activity in biological samples after ingestion of green and black tea (17, 18). Although the primary biological response observed with these methods was probably due to the absorption of flavonoids, any polyphenol dimers, thearubigens, or their metabolites that were absorbed also may have contributed to the response.

Table I. Current Status of Routine Methodology for the Measurement of Tea Polyphenols

Polyphenol fraction	Sample source	
	Tea	Plasma/urine
Total polyphenols	Reducing activity	Reducing activity
Flavonoids	HPLC/UV[a]	Fractionation/ colorimetry HPLC/FL[b] HPLC/EC[c]
Theaflavins	HPLC/UV	nd[d]
Other dimers	nd	nd
Thearubigens	Total reducing activity/ subtraction of known polyphenols	nd

[a] High-performance liquid chromatography with ultraviolet and visible detection.
[b] High-performance liquid chromatography with fluorescent detection.
[c] High-performance liquid chromatography with high sensitivity electrochemical detection.
[d] Routine measurement system has not been developed.

Microorganisms commonly found in the gastrointestinal tract of monogastric animals (humans included) readily cleave the rings of simple polyphenolics and may also metabolize polyphenol condensation products (see Ref. 29 for review). Microbial enzymes have affinity for specific double bond–functional group combinations; therefore, the products that result are dependent upon the subclass of polyphenols (flavanols, flavonols, etc.) that are metabolized. In general, the resulting metabolites are small molecular weight phenolic acids, many of which may be absorbed and further metabolized in the body. Since less than 5% of the intact polyphenols are absorbed after a dose of tea (15), the monitoring of polyphenol metabolic products may be another reasonable approach to elucidation of the metabolism of tea polyphenols. Gross *et al.* developed an HPLC system to monitor the levels of three metabolites of quercetin (3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, and 4-hydroxy-3-methoxyphenylacetic acid) in the urine of human subjects (30). Although these investigators did not test the effects of tea administration on the levels of these metabolites, they did observe increased urinary levels of these compounds when subjects consumed high levels of fruits and vegetables, foods that contain substantial amounts of quercetin. The literature lacks analogous studies that have investigated the response of urinary levels of metabolites of catechin after consumption of green or black tea. However, Shahrzad and Bitsch developed an HPLC system for the measurement of gallic acid, a prominent phenolic acid of tea, and several of its metabolites in human plasma and urine (31). This system has been applied to monitor the

metabolism of pharmacological doses of gallic acid (31) but not the levels commonly observed in tea beverages or foods.

Summary

The current status of analytical methodology for the routine measurement of polyphenols in tea, tea extracts, and biological samples is summarized in Table I. In general, total polyphenol content is currently measured employing methodology that is based on reducing activity. Several HPLC systems with detectors that, collectively, have wide ranges in sensitivity have been developed for analysis of individual flavonoids in tea and biological samples and for theaflavins in tea. Catechins also have been measured in plasma by solid phase extraction, addition of a chromophore, and quantified colorimetrically. Except for theaflavins in tea, routine and robust methods for the measurement of polyphenol condensation products (dimers and thearubigens) in tea and biological samples have not been developed. Only a single HPLC procedure has been assembled for monitoring the metabolic products of quercetin in urine of human subjects.

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