Development of a Competition Assay for the Evaluation of the Binding of Human Parotid Salivary Proteins to Dietary Complex Phenols and Tannins Using a Peroxidase-Labeled Tannin

James R. Bacon* and Michael J. C. Rhodes

Biochemistry Department, Institute of Food Research, Norwich Research Park,
Colney, Norwich NR4 7UA, United Kingdom

Proline-rich proteins (PRP) are secreted by herbivores, including humans, in their parotid saliva into the oral cavity. These PRP have a high affinity for dietary complex phenols and tannins (CPT). The stable complexes formed may then pass through the digestive tract and are excreted. It is postulated that such complexes may modulate both the antinutritional effects and beneficial properties of CPTs. A novel competitive tannin−salivary protein binding assay is described that allows the detailed investigation of binding of tannins to salivary proteins. The assay is based on the competition between an enzyme-labeled tannin [(−)-epigallocatechin conjugated to horseradish peroxidase via a linker molecule] and a test tannin to bind to parotid salivary protein immobilized in the wells of a polystyrene microtiter plate. The binding affinities of a series of flavan-3-ol monomers from tea and a commercial tannic acid preparation were determined. Major differences in the binding affinities of the compounds tested to the salivary proteins were observed. A relationship between the level of tannin galloylation and the affinity of binding to parotid salivary protein is demonstrated.

INTRODUCTION

Plants synthesize a diverse group of phenolic compounds that vary in molecular complexity from simple phenolic acids to lignin and the complex polymers of the condensed and hydrolyzable tannins (Haslam, 1989). There are two main types of tannins. Condensed tannins are dimers, oligomers, and polymers of variously substituted flavan-3-ols, such as catechin and epicatechin, and are linked through carbon 4, commonly to carbon 8 but less commonly to carbon 6. Condensed tannins are found in most fruits and legumes and in beverages such as tea, wine, beer, and cider, making important contributions to their flavor. Hydrolyzable tannins are characterized by a polyhydric alcohol core, usually glucose, the hydroxyl groups of which are fully or partially esterified by gallic acid or hexahydroxy-diphenic acid. Hydrolyzable tannins are particularly abundant in pomegranate, persimmon, chestnuts, and fruits of the Rosaceae.

Tannins have been demonstrated to have various properties that may be beneficial or detrimental to health. Although evidence from animal studies and epidemiology is in some cases contradictory and there is limited knowledge of their bioavailability, particularly of the more complex tannins, there is little doubt that they are physiologically important compounds. Tannins are able to form stable complexes with proteins and polysaccharides, and such complexation is probably fundamental to many observed biological activities. This property also contributes to the sensation of astringency in the mouth when these compounds are consumed by mammals. Black and green tea extracts have been shown to inhibit the formation of skin, lung, and forestomach cancers, induced by UVB light and chemi-cal carcinogens, in rodents (Wang et al., 1992, 1994). Plant polyphenols are acknowledged as powerful biological antioxidants (Rice-Evans et al., 1995; Hagerman et al., 1998), and there is much interest in the potential benefits or risks but the data are largely from in vitro experimentation and difficult to interpret in terms of human physiological response.

On digestion, the first interaction of food is with saliva. Herbivores produce proline-rich proteins (PRP), in their saliva, that have a very high affinity for tannins relative to other proteins (Hagerman and Butler, 1981). In humans, PRP account for ∼70% of the protein in parotid saliva (Bennick, 1982), the remainder being mainly α-amylase. PRP in saliva may comprise a substantial number of isoforms but can be subdivided into three classes: acidic, basic, and glycosylated, with proline contents ranging between 15 and 45%. Little is known of the tannin binding properties of these different classes, and similarly little is known of structural features of complex phenols and tannins that may be important in binding to salivary PRP, but factors such as the degree of polymerization have been implicated (Baxter et al., 1997).

Protein−tannin interactions are normally measured using assays based on the competition of a test protein with a standard labeled protein to bind and coprecipitate with a tannin (Hagerman and Butler, 1981; Waterman and Mole, 1994). Although salivary proteins and tannins readily form complexes, such interactions do not in themselves necessarily lead to precipitation (Yan and Bennick, 1995), and precipitation assays may be therefore inappropriate in some circumstances. Protein−tannin interactions have been likened in some respects to the binding of antigens to antibodies. We have therefore investigated whether the techniques

used in immunochemistry can be applied to tannin–protein interactions and have developed an assay based on the principle of a competitive ELISA. This assay has been applied to a series of flavan-3-ol monomers found in tea and a commercial sample of tannic acid, a mixture of hydrolyzable tannins.

MATERIALS AND METHODS

Materials. All chemicals were of AnalaR grade and purchased from BDH, Poole, U.K. 1,4-Butanediol diglycidyl ether, tannic acid, peroxidase (horseradish, HRP), and all other proteins purchased were from Sigma Chemical Co., Poole, U.K., with the exception of α-amylase (human saliva), which was obtained from Fluka Chemicals, Gillingham, U.K. Epigallocatechin and all other catechin monomers were provided by Unilever Research, Colworth, U.K. Water was purified by a Millex Q-plus system (Millipore, Watford, U.K.).

Human Parotid Saliva Collection. Parotid saliva (total, 65 mL) was collected from five healthy, nonsmoking volunteers (two females and three males, aged 25–45 years) using parotid saliva collection cups manufactured at IFR to the specifications described by Stephen and Speirs (1976). Saliva flow was induced by applying small quantities of lemon juice onto the volunteers' tongues. After collection, EDTA was added to the saliva to give a final concentration of 5 mM. The bulked saliva samples were dialyzed exhaustively against water at 2 °C, lyophilized, and stored at −20 °C.

Synthesis of Peroxidase–Tannin Conjugate. Conjugates were prepared using adaptations of the method of Lommen et al. (1995) in which phenolic compounds can be conjugated to proteins via a linker molecule, 1,4-butanediol diglycidyl ether. A peroxidase-linker molecule (HRP-L) was prepared first by dissolving 22.5 mg of HRP in 0.5 mL of water, and the pH was adjusted to 10.8 with ~5 μL of 0.5 M NaOH. Fifty microliters of a solution of 1,4-butanediol diglycidyl ether in water (20 mg/mL) was added and the mixture stirred at room temperature, under a stream of nitrogen for 24 h. The HRP-L was recovered as a red/brown fraction (1.6 mL) from the reaction mixture on a PD10 desalting column (Amersham Pharmacia Biotech, St. Albans, U.K.) pre-equilibrated in 100 mM NaCl. Subsamples of this HRP-L solution were stored at −20 °C for use as a control in the development of the protein–tannin binding assay.

The peroxidase–tannin conjugate was prepared by transferring the product of one HRP-L preparation as described above into 20 mM Tris-HCl, pH 8.5, using a PD10 column. The red/brown protein fraction from the eluate (equivalent to 22.5 mg of HRP in 2 mL) was added to 10 mg of epigallocatechin (EGC) and stirred at room temperature, under a stream of nitrogen, for 48 h. The conjugate (HRP–EGC) was recovered from the mixture by chromatography on a PD10 column pre-equilibrated in 20 mM NaH_2PO_4/150 mM NaCl, pH 7.0. The HRP–EGC solution (final concentration = 10 mg/mL) was stored at −20 °C.

Protein concentrations of conjugate solutions were determined on microtiter plates using a bicinchoninic acid kit (Sigma) according to the manufacturer's instructions. Assay volume was 0.2 mL, and standard curves were prepared by the addition of solutions containing 0–5 μg of HRP. The range of linearity of HRP activity was determined on a microtiter plate by the addition of a series of dilutions of HRP–EGC up to 0.5 ng per well in a volume of 25 μL. HRP activity was determined by the addition of 200 μL of a commercial peroxidase chromogen as described below.

Tannin–Protein Binding Competition Assay. Microtiter plates were coated with parotid saliva protein as follows: Stock solution (1 mg/mL) and working strength solution (0.1 μg/mL) of lyophilized protein were prepared in 50 mM $NaHCO_3$ buffer, pH 9.6. Two hundred microliter aliquots of the working strength protein solution were pipetted into the center 60 (6 rows × 10 columns) wells of a 96-well polystyrene microtiter plate (Nunc Maxisorp), covered, and incubated at 2 °C overnight. For each experiment, a blank was similarly
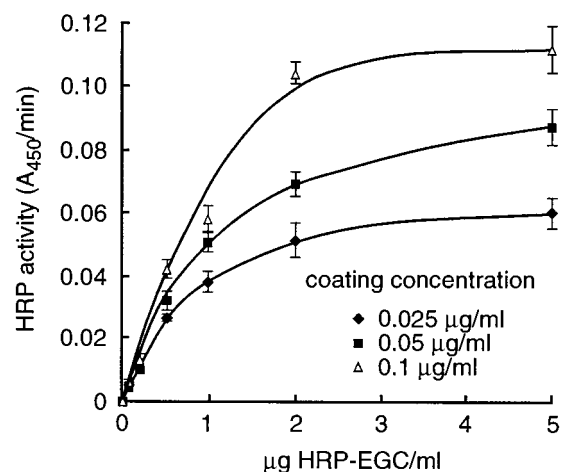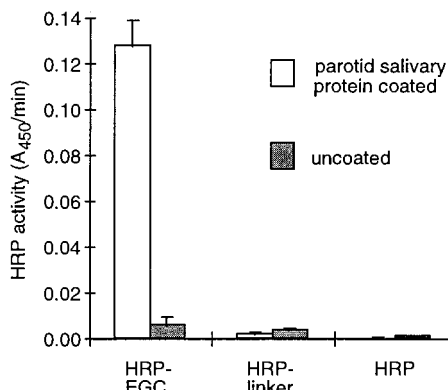

Figure 1. Saturation binding curves of HRP–EGC solutions on microtiter plates coated from different concentrations of parotid salivary protein (as indicated in the key). Level of binding was measured as HRP activity. All other conditions are as for standard assay conditions as described under Materials and Methods. Each point represents mean ± SD ($n \geq 6$ wells).

prepared by incubating a plate with 50 mM $NaHCO_3$ buffer, pH 9.6. All plates were then washed three times with 20 mM NaH_2PO_4, 150 mM NaCl, and 0.05% Tween 20, pH 6.0 (wash buffer), using a Wellwash 5000 microplate washer. The coated wells were then loaded with 200 μL of HRP–EGC (2 μg/mL) in 20 mM NaH_2PO_4, 150 mM NaCl, and 0.05% Tween 20, pH 8.0, containing, in sequential columns, serial dilutions from 0 to 100 μg/mL of the tannin under test (i.e., giving six replicates of each condition). The plates were incubated for 2 h at 37 °C and then washed five times with wash buffer as described above. To each well was added 200 μL of a 1:1 mix of a commercial peroxidase chromogen solution and substrate buffer solution (Vétoquinol, Bicester, U.K.). Plates were developed at room temperature for 5 min, and the reaction was stopped by the addition of 50 μL of 2 M H_2SO_4. The absorbance values of the contents of all wells were measured at 450 nm on a microplate reader (Dynatech MR5000). The absorbance values, minus those of the blank (uncoated) plate, were plotted against concentration of test tannin, and the molarity of test tannin required to displace 50% of the HRP–EGC conjugated to the protein coating on the microtiter plate relative to the control value (no tannin) was calculated. The percentage of HRP–EGC displaced by a specific concentration of tannin was also calculated. These experimental conditions were varied during the development of the assay. Such variations are described for the individual experiments under Results and Discussion.
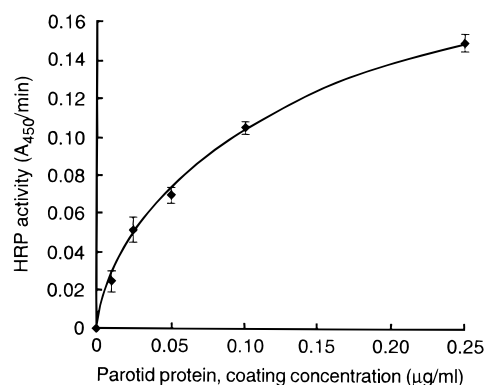
RESULTS AND DISCUSSION

Competition Assay Development and Validation. Saturation curves (Figure 1) were used to determine appropriate levels of parotid salivary protein for coating of the wells (0.1 μg/mL) and for binding of HRP–EGC (2 μg/mL). Under these conditions, the level of HRP–EGC just saturates the available protein binding sites and hence maximizes the opportunity for competition between the test and labeled tannins while giving a conveniently measurable level of bound enzyme activity. Analysis of the synthesized HRP–EGC conjugate showed an enzyme activity/concentration relationship linear up to the addition of least 0.5 ng of HRP–EGC/well (data not shown), giving activity levels of 0.55 (A_{450}/min). The level of HRP activity associated with the bound HRP–EGC detected under the finally established assay conditions, in the absence of competing tannin,
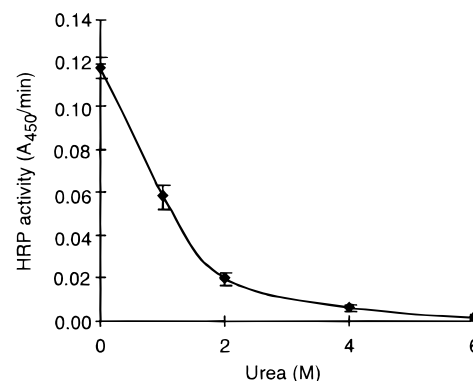
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Figure 2. Comparison of binding of HRP-labeled tannin (HRP−EGC), of HRP conjugated with linker (HRP-L), and of horseradish peroxidase (HRP) to microtiter plates coated from parotid salivary protein solutions and uncoated plates using standard assay conditions. Level of binding was measured as HRP activity. Concentration of HRP and conjugate solutions = 2 μg/mL. Each point represents mean ± SD ($n \geq 6$ wells).

Figure 3. Effect of parotid protein coating concentration on binding of enzyme-labeled tannin (HRP−EGC) from 2 μg/mL solution using standard assay conditions. Level of binding was measured as HRP activity. Each point represents mean ± SD ($n \geq 6$ wells).

Figure 4. Effect of the addition of urea to competition buffer on the binding of enzyme-labeled tannin (HRP−EGC) to microtiter plates coated from parotid saliva protein solutions using standard assay conditions. Level of binding was measured as HRP activity. Each point represents mean ± SD ($n \geq 6$ wells). Suitable controls were carried out to show that urea treatment did not solubilize salivary protein from the coated surfaces and did not inhibit HRP activity.

was typically equivalent to that produced by 0.15 ng of HRP−EGC. The time required for optimal binding of HRP−EGC to the parotid salivary protein was also investigated and was found to be relatively long, compared to that recommended for many tannin−protein precipitation assays [15 min at 37 °C (Hagerman and Butler, 1981); 15 min at room temperature (Waterman and Mole, 1994)], with 2 h at 37 °C being required to reach equilibrium. Only 30% of this maximum level was detected after 15 min and 85% after 1 h (data not shown).

Figure 2 shows the relative binding of HRP−EGC, of HRP conjugated with linker molecule (1,4-butanediol diglycidyl ether), and of HRP alone, in the absence of any test competing tannin, both to plates coated with parotid salivary protein and to uncoated blank plates. The blank values, that is, nonspecific binding of HRP conjugates to the surface of the polystyrene plate in the absence of salivary proteins, were reduced to a low level by the addition of Tween 20 to wash and competition buffers to block such binding sites, a protocol that is commonly used in ELISA assays to overcome problems of nonspecific antibody binding (Wilkins Stephens et al., 1995). The coated plates treated with HRP−EGC gave a 50-fold higher level of HRP binding compared with those treated with the HRP linker, and no binding was detected with HRP alone, thus demonstrating that binding of HRP−EGC was strongly dependent on the conjugation with the tannin. On uncoated plates, the level of binding of HRP linker was not significantly different from that observed on coated plates, and any binding resulting from the attachment of the linker molecule to HRP can therefore be attributed to residual nonspecific interactions and not associated with binding to parotid salivary protein. A low level of nonspecific binding was also observed with HRP−EGC on uncoated plates, but this amounted to <4% of the binding observed on coated plates. The effect of varying the parotid saliva coating solution concentration on the level of HRP−EGC binding under our standard assay conditions demonstrates a clear dose/response relationship (Figure 3). The type of binding observed between the HRP−EGC conjugate and the salivary proteins coated onto plates was investigated by performing the assay in the presence of a series of concentrations of urea (Figure 4), an agent known to disrupt hydrophobic interactions (Creighton, 1984). The addition of, for

example, 4 M urea to the competition buffer reduced the level of HRP−EGC binding by 95%. The treatment of coated wells with urea prior to the addition of the HRP−EGC conjugate did not reduce subsequent conjugate binding to the wells after the urea was washed out (data not shown). Thus, the inhibitory effect on HRP−EGC binding to the salivary protein cannot be due to an effect on salivary protein solubilization. Similarly, treatment of HRP with urea, at the concentration used, was shown not to affect its activity. The major mode of binding between tannins and proteins is thought to involve primarily hydrophobic interactions, with hydrogen bonding taking a secondary role (Luck et al., 1994); thus, the sensitivity to urea suggests that binding of the HRP−EGC conjugate to the salivary protein occurs by the same mechanism involved in the interactions of proteins with free tannin.

HRP−Conjugate Binding Specificity for Human Parotid Salivary Proteins. Results in Figure 5 demonstrate the specificity of HRP−EGC binding to parotid salivary protein coated plates compared with plates coated with a number of other proteins known to bind well to polystyrene surfaces (Pesce et al., 1977; Cantarero et al., 1980; Sorensen and Brodbeck, 1986). The level of binding of the HRP−EGC to salivary proteins was at least an order of magnitude higher than with any other protein tested and with most other

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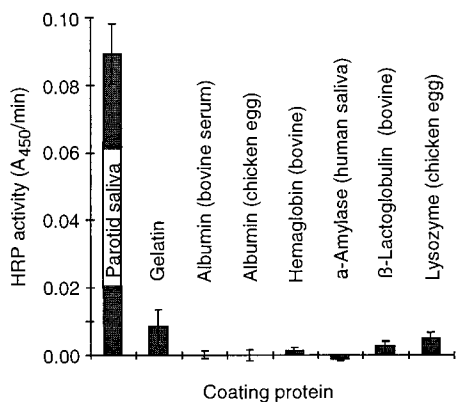
Figure 5. Comparison of binding of enzyme-labeled tannin (HRP–EGC) to microtiter plates coated from different protein solutions (0.1 μg/mL) using standard assay conditions. Level of binding was measured as HRP activity. Each point represents mean $\pm$ SD ($n \geq 6$ wells).

proteins showing only a relatively low level of binding. Cantarero et al. (1980) reported on the binding of chicken egg albumin and bovine serum albumin to polystyrene tubes and showed that 50 and 25%, respectively, of these proteins could be bound from a 0.1 μg/mL solution in NaHCO$_3$ buffer, pH 9.6, and neither of these proteins gave a detectable level of HRP–EGC binding in our assay. Among the proteins that also failed to bind the HRP–EGC conjugate was human salivary α-amylase, which is the major, non-PRP protein component of parotid saliva. A low level of binding (<10% of that with parotid salivary protein) was, however, observed on plates coated with gelatin, and this may be related to its relatively high proline content [typically 16% (Long, (1961)]. In protein binding competition experiments, Hagerman and Butler (1981) similarly showed that both gelatin and rat parotid proline-rich protein had higher affinities for sorghum tannin than any other proteins tested. It is hence reasonable to conclude from the results obtained that the HRP–EGC conjugate has a particularly high affinity for salivary PRP and that such binding is derived from the tannin part of the conjugate. Salivary PRP have other physiological roles, such as in oral homeostasis (McArthur et al., 1995), but the preferential binding of PRP to tannins indicates that they may act as the first line of defense against dietary tannins. The extent to which dietary tannins are sequestered in such complexes during mastication and whether they can pass directly through the digestive tract and are then excreted is unknown. However, such complexation is likely to modulate any biological activities whether deleterious or beneficial to health.

Application of Competitive Binding Assay to Tannins. Figure 6 shows binding curves obtained from the application of the assay to the assessment of the binding to parotid saliva protein of condensed tannin monomers (flavan-3-ols) obtained from tea and a commercial tannic acid (Sigma), which comprises a mixture of high molecular weight hydrolyzable tannins, principally a range of polymers from mono- to octa-O-galloyl-D-glucose. Values for the molarity of tannin required to be added to displace 50% of the HRP–EGC binding ($A_{0.5}$) and the amount of HRP–EGC displaced by 10 μg/mL of each tannin have been extracted from such curves and are shown in Table 1. These latter values show that, with the addition of the most strongly binding compounds, 90–95% of the HRP–EGC can be readily

◆ catechin ■ ECG
□ EC ▲ (-) GCG
△ EGC ● EGCG
○ tannic acid
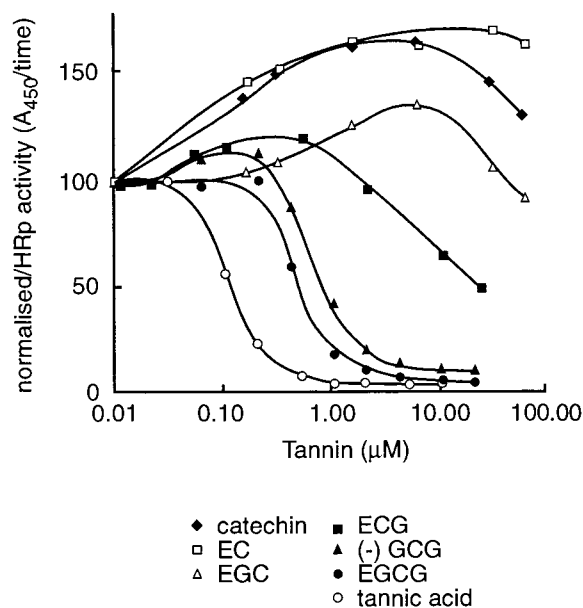
Figure 6. Competitive binding of test tannins with enzyme-labeled tannin (HRP–EGC) to parotid salivary protein coated microtiter plates using standard assay conditions. Level of binding was measured as HRP activity. Data are normalized with respect to control activity with no competing tannin added (value given = 100). This control value was typically equivalent to an enzyme activity of 0.15 (A_{450}/min). Each point represents mean $\pm$ SD ($n \geq 6$ wells).

Table 1. Binding Affinities of Tannins to Human Parotid Salivary Protein

tannin	$A_{0.5}$, μM	% HRP–EGC displaced by 10 μg/mL tannin
tannic acid[a]	0.12	94.8
(+)-catechin	na[b]	0
(−)-epicatechin	na	0
(−)-epigallocatechin	na	0
(−)-epicatechin gallate	25.3	51.8
(−)-gallocatechin gallate	0.92	89.3
(−)-epigallocatechin gallate[c]		
mean	0.54	93.2
SD	0.07	2.0

[a] Molecular weight of tannic acid calculated as pentagalloyl-glucose. [b] na, not achieved at concentration of 100 μg/mL ($\cong$350 μM catechin). [c] Mean $\pm$ SD of four assays on separate days.

displaced, demonstrating that a full competition is taking place with the competing tannin occupying the majority of the available binding sites on the parotid saliva plate coating. Determination of the $A_{0.5}$ value for (−)-epigallocatechin gallate gives a mean of 0.54 $\pm$ 0.07 μM ($n = 4$), and >90% of the HRP–EGC was displaced in each experiment, thus demonstrating the reproducibility of the assay. The compounds tested can be ranked in order of their ability to displace HRP–EGC binding to salivary parotid proteins and hence their own degree of ability to bind to parotid salivary protein as follows: tannic acid > (−)-epigallocatechin gallate > (−)-gallocatechin gallate > (−)-epicatechin gallate > (−)-epigallocatechin = (−)-epicatechin = (+)-catechin. The latter three compounds failed to displace 50% of the bound HRP–EGC at the highest concentrations tested of 100 μg/mL ($\cong$350 μM catechin). Interestingly, the addition of EGC to the assay only caused significant displacement of binding of HRP–EGC, that is, self-competition with the tannin conjugated to the HRP, at concentrations beyond 50 μM. The protein binding properties of EGC appear, therefore, to be modified

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Ricardo-da-Silva, J. M.; Cheynier, V.; Souquet, J.-M.; Moutounet, M.; Cabanis, J.-C.; Bourzeix, M. Interaction of grape seed procyanidins with various proteins in relation to wine fining. *J Sci. Food Agric.* **1991**, *57*, 111−125.

Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. M. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* **1995**, *22*, 375−383.

Sorensen, K.; Brodbeck, U. Assessment of coating-efficiency in ELISA plates by direct protein determination. *J. Immunol. Methods* **1986**, *95*, 291−293.

Stephen, K. W.; Speirs, C. F. Methods for collecting individual components of mixed saliva: the relevance to clinical pharmacology. *Br. J. Clin. Pharmacol.* **1976**, *3*, 315−319.

Wang, Z. Y.; Hong, J. Y.; Huang, M. T.; Reuhl, K. R.; Conney, A. H.; Yang, C. S. Inhibition of *N*-nitrosodiethylamine- and 4-(methylnitrosamino)-1-(3-pryridyl)-1-butanone-induced tumorigenesis in A/J mice by green tea and black tea. *Cancer Res.* **1992**, *52*, 1943−1947.

Wang, Z. Y.; Huang, M. T.; Lou, Y. R.; Xie, J. G.; Reuhl, K. R.; Newmark, H. L.; Ho, C. T.; Yang, C. S.; Conney, A. H. Inhibitory effects of black tea, green tea, decaffeinated black tea, and decaffeinated green tea on ultraviolet B light-induced skin carcinogenesis in 7,12-dimethylbenz[*a*]anthracene-initiated SKH-1 mice. *Cancer Res.* **1994**, *54*, 3428−3435.

Waterman, P. G.; Mole, S. In *Analysis of Phenolic Plant Metabolites*; Blackwell: London, U.K., 1994.

Wilkins Stephens, P.; Hansberry, M. R.; Kelso, D. M. Assessment of adsorption and adhesion of proteins to polystyrene microwells by sequential enzyme-linked immunosorbant assay analysis. *Anal. Biochem.* **1995**, *225*, 197−205.

Yan, Q.; Bennick, A. Identification of histatins as tannin-binding proteins in human saliva. *Biochem. J.* **1995**, *311*, 341−347.