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SCIENCE DIRECT
Food Chemistry 100 (2007) 1704–1712
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Flavonoids from a grape seed extract interact with digestive secretions and intestinal cells as assessed in an in vitro digestion/Caco-2 cell culture model

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Received 14 March 2005; received in revised form 22 September 2005; accepted 23 October 2005

Abstract

Although the bioactivity of flavonoids is related to their bioavailability, little is known about pre-absorption events in the gastrointestinal tract and their possible interactions with digestive constituents and intestinal cells. Using an in vitro digestion/Caco-2 cell culture model, we investigated the effect of digestive secretions on the stability of (+)-catechin (CAT), (−)-epicatechin (EC) and B2 and B3 dimers from a procyanidin-rich grape seed extract (GSE). The availability of phenolic compounds was not affected by salivary and gastric incubations but decreased during intestinal digestion in the absence of Caco-2 cells due to interactions with pancreatic proteins, unmasked by acetonitrile extraction. Then, in the presence of cells, about 43.9% of CAT, 85.3% of EC and all dimers disappeared at the end of 2 h of intestinal incubation. The stability of all compounds at intestinal pH was demonstrated as well as interactions with proteins, associated with a decrease of some cells enzyme activities, e.g., alkaline phosphatase (−79.8%), sucrase-isomaltase (−60.9%) and aminopeptidase N (−60.7%). Moreover, no compounds were detected in the basal compartment of transwells or in cell monolayers.

Keywords: Procyanidin-rich grape seed extract; Caco-2 cells; In vitro digestion; Proteins interactions

1. Introduction

Flavonoids have been the focus of research interest for many years, as they can help prevent diseases associated with oxidative stress. Flavonoids belong to a large group of naturally occurring plant polyphenols that are consumed daily in large amounts. Such compounds are principally found in teas (Aherne & O'Brien, 2002; Arts, Van de Putte, & Hollman, 2000; Khokhar & Magnusdottir, 2002), red wines (Arts et al., 2000; Landrault et al., 2001; Waterhouse & Teissèdre, 1997), cacao products (Arts, Hollman, & Kromhout, 1999; Arts et al., 2000; Hammerstone, Lazarus, & Schmitz, 2000), and also in a lot of fruits such as apples, berries and grapes, which are particularly rich in flavanols and procyanidins (Fuleki & Ricardo da Silva, 1997). Epidemiological studies have revealed a relationship between flavonoid consumption and the prevention of diseases such as cancer or coronary heart diseases (Cook & Sammam, 1996). Many in vitro studies have characterized flavonoids as powerful antioxidants capable of efficient scavenging of both reactive oxygen and nitrogen species (Bagchi et al., 2003; Burda & Oleszek, 2001; Rice-Evans, Miller, & Paganga, 1996).

However, it is now clear that the ultimate antioxidant potential of flavonoids and their resulting in vivo bioactivity is dependent on their absorption, metabolism, distribution and excretion within the body after ingestion. Many studies have thus been conducted in recent years to evalu-

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doi:10.1016/j.foodchem.2005.10.016

ate their bioavailability, and various chemical forms of flavonoids and their metabolites have been detected in plasma and urine (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005; Williamson & Manach, 2005). Although there could possibly be interactions between flavonoids and digestive secretion constituents – namely saliva, gastric and pancreatic juices – which would affect flavonoid availability, few studies have focused on the behavior of flavonoids throughout the digestive tract. Indeed, it is well known that phenolic compounds can have strong affinities with proteins and particularly with human salivary proline-rich proteins (PRPs) and histatins (de Freitas & Mateus, 2001; Lu & Bennick, 1998; Sarni-Manchado, Cheynier, & Moutounet, 1999; Wroblewski, Muhandiram, Chakrabartty, & Bennick, 2001) to form both non-covalent and covalent associations according to the phenolic compound size. Other studies (Lu & Bennick, 1998; Wroblewski et al., 2001) have demonstrated that insoluble complexes, which result from interactions between proteins and tannins, are stable throughout the digestive tract. However, little is known about interactions between low molecular weight (MW) flavonoids and proteins. According to Arts et al. (2002), (+)-catechin (CAT) and (−)-epicatechin (EC) are able to bind β-casein by the way of proline residues. de Freitas and Mateus (2001) have shown that CAT, EC and B2 and B3 dimers can interact with PRPs. Rhon, Rawel, and Kroll (2002) noted a loss of pancreatic α-amylase and trypsin activities in the presence of phenolic compounds. Moreover, polyphenols seem to have affinities for enterocyte brush border enzymes (Tebib, Rouanet, & Besançon, 1995; Welsch, Lachance, & Wasserman, 1989).

This study was aimed at investigating the behavior of flavan-3-ol monomers and procyanidin dimers from a procyanidin-rich grape seed extract (GSE) through the digestive tract and to assess some possible interactions between these compounds and proteins, in order to evaluate the consequences on their bioavailability. For this study, we used an in vitro digestion/Caco-2 cell culture model adapted from Glahn, Wien, Van Campen, and Miller (1996) to mimic the physiological conditions of flavonoid intake. GSE was thus subjected to successive incubations in saliva, gastric and pancreatic media, with the last incubation conducted in the presence of a Caco-2 cell monolayer.

Hence, (+)-catechin and (−)-epicatechin, B2 and B3 dimers from GSE were studied. The Caco-2 cell line is a well-accepted model of intestinal absorption (Boulenc, 1997; Hidalgo, Raub, & Borchard, 1989; Hillgren, Kato, & Borchardt, 1995) and was previously used to study the transport of flavonoids (Deprez, Mila, Huneau, Tome, & Scalbert, 2001; Galijatovic, Otake, Walle, & Walle, 1999; Liu & Hu, 2002; Vaidyanathan & Walle, 2001) but also of minerals (Glahn, Cheng, & Welch, 2002; Planes et al., 2002; Puyfoulhoux et al., 2001) and drugs (Biganzoli, Cavenaghi, Rossi, Brunati, & Nolli, 1999; Jezyk et al., 1999).

2. Materials and methods

2.1. Materials

Grape seed extract (GSE) was obtained from La Gardonnenque S.C.A (Cruviers Lascours, France) and contained approximately 67.4% procyanidins, with 13.5% of them being (+)-catechin (CAT), (−)-epicatechin (EC) and B1, B2, B3 and B4 dimers (according to the supplier). The GSE concentration used in this study was equivalent to the per-meal consumption of 250 mL of red wine (1–5 g/l of total polyphenols) for an adult subject, corresponding to a polyphenol intake of 0.25–1.25 g. The volume of total digestive secretions in man is estimated to be 10 l/day, i.e., around 3 l/meal. Ingested polyphenols may thus be diluted in this secretion volume and reach intestinal cells at a concentration range of 85–400 mg/l. A final GSE concentration of 0.3 g/l was therefore chosen for this experiment.

2.2. Cell culture

Caco-2 cells from human colon adenocarcinoma were obtained from the American Type Culture Collection and used in experiments between passages 40 and 49. Caco-2 cells were maintained and expanded in 75 cm^2 flasks at 37 °C in an atmosphere of 5% CO_2/95% air at constant humidity and in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 10% (v/v) fetal calf serum, 2% (v/v) L-glutamine, 1% antibiotic antimycotic solution and 1% nonessential amino acids and was changed daily. At confluence, i.e., when a monolayer was formed, cells were harvested by treatment with a solution containing 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA), thoroughly washed and resuspended in supplemented growth medium. All products were purchased from Invitrogen SARL (Cergy Pontoise, France). For the experiments, Caco-2 cells were seeded at 50,000 cells/cm^2 density (Glahn et al., 1996) in permeable polyester transwell cell culture inserts (pore size 0.4 μm; Corning Costar Science Products, Brumath, France) to mimic the physiological conditions of an intestinal barrier. The filters were located in 6-well plates separating apical from basolateral sides. The experiments were conducted from 18 to 21 days postconfluency. The integrity of the cell monolayer was examined by determining the transepithelial passage of phenol red (a pH-indicator) which, according to Ekmekioglu, Ekmekioglu, and Marktl (2000), is only transported paracellularly.

2.3. In vitro simulated digestion of GSE

This was performed according to previous studies (Glahn et al., 2002; Planes et al., 2002; Puyfoulhoux et al., 2001). Incubations in salivary, gastric and intestinal solutions were conducted on a rocking platform shaker (Rotomix, Bioblock, Illkirch, France) in an incubator at

37 °C. All enzymatic solutions such as α-amylase from human saliva (1000–1500 U/mg protein), porcine pepsin (800–1000 U/mg protein), pancreatin (activity, 4× USP specifications) and bile extract (glycine and taurine conjugates of hyodeoxycholic acids and other bile salts) were purchased from Sigma (Saint Quentin Fallavier, France).

GSE was added to artificial saliva to reproduce the salivary step. The pH was adjusted to 6.9 with 1 N HCl and α-amylase was added. The samples were incubated for 10 min at 55 oscillations/min. The pH was adjusted to pH 2 with 1 N HCl and 0.05 ml of pepsin (25 mg/ml in 0.1 N HCl) was added per milliliter of sample to simulate a gastric juice. Incubation was performed for 60 min at 55 oscillations/min. Finally, to reproduce the intestinal medium, the acidity was raised to pH 6 by dropwise addition of 1 M $NaHCO_3$ and 0.25 ml of pancreatin-bile extract solution (2 g/l of pancreatin and 12 g/l of bile extract in 0.1 M $NaHCO_3$) was added per milliliter of sample. The pH was adjusted to 7 with NaOH and the sample was diluted (2:3) with a 120 mM NaCl/5 mM KCl solution. Pancreatic incubation was conducted in the presence of Caco-2 cells to mimic the intestinal digestion step. Culture medium was thus removed from each well just before the experiment and replaced in the upper compartment by 1.5 ml of predigested sample diluted with culture medium (3:5). Culture medium was also added (2 ml) in the lower compartment. Incubation was performed for 120 min at 6 oscillations/min and CAT, EC and B2 and B3 dimer quantities were assessed both in apical and basal medium and in the Caco-2 cell monolayer.

In order to evaluate the stability of the phenolic compounds through the intestinal incubation period and their behavior in the presence of Caco-2 cells, the GSE sample (0.3 g/l) was also subjected to the in vitro digestion previously described in the absence of Caco-2 cells. Pancreatic incubation was thus performed in tubes and phenolic compounds were analyzed at the end of the 2 h incubation period.

2.4. Protein/flavonoid interactions

In order to investigate some possible masking of phenolic compounds by proteins through digestion, GSE was subjected to the in vitro digestion process described previously and proteins were extracted just before the HPLC analysis, using acetonitrile, according to Lee, Prabhu, Meng, Li, and Yang (2000). Briefly, samples were mixed with an equal volume of acetonitrile, vortexed for 30 s and centrifuged at 16,000*g* for 10 min. The resulting supernatant solutions were subjected to HPLC analysis.

2.5. HPLC analysis of phenolic compounds

A Hewlett-Packard model 1090 HPLC with three low pressure pumps and a diode array UV detector coupled to an Hewlett-Packard Chem station was used for solvent delivery system and detection. A Hewlett-Packard column packed with Nucleosil 100 C18 (250 × 4 mm, 5 μm particle size) was used for the stationary phase with a 0.7 ml/min flow rate. The solvents used for separation (Lameula-Raventos & Waterhouse, 1994) were as follows: solvent A: 50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B: 20% (v/v) A with 80% (v/v) acetonitrile; solvent C: 200 mM orthophosphoric acid adjusted with ammonia to pH 1.5. Elution was performed using a previously described gradient (Carando, Teissèdre, Pascual-martinez, & Cabanis, 1999).

2.6. Enzymatic assays

To evaluate the impact of phenolic compounds or their oxidation products on intestinal brush border cells, the activities of some hydrolases, such as alkaline phosphatase, aminopeptidase N and a disaccharidase (sucrase-isomaltase), were assessed at the end of the in vitro digestion of 0.3 g/L GSE performed in the apical compartment of transwells. We also studied the activity of a cytoplasmic enzyme, i.e., lactate dehydrogenase (LDH), in both cells and apical medium. For comparison, enzymatic activities were also assayed on control Caco-2 cells, i.e., without contact with digested GSE. After removal of the apical solution, the cell monolayer was washed twice with prewarmed (37 °C) PBS, scraped, harvested in 2 ml of water and sonicated for 30 s. All assays were performed with a microtiter plate reader (Dynex Technologies, Grafton, OH, USA).

Sucrase-isomaltase was assayed according to Messer and Dahlqvist (1966), alkaline phosphatase according to Bessey, Lowry, and Brock (1946) and aminopeptidase N according to Maroux, Louvard, and Baratti (1973). LDH was assayed using a standard ultraviolet spectrophotometric kit (SIGMA, method 228-UV). Specific enzyme activities were determined (U/mg of protein: one unit is defined as the activity that hydrolyses 1 μmol of substrate per minute under the experimental conditions) and finally expressed as a percentage of specific control cell activities. The Caco-2 cell protein content was assayed by the bicinchoninic acid (BCA) method (Smith et al., 1985) with bovine serum albumin (BSA) as standard.

2.7. Statistical analysis

For the enzymatic assays, three wells were run and each assay was repeated three times. Data from each experiment were averaged ($n = 9$) and this average value was the data point used in the statistical analysis. For phenolic compound analysis, each experiment was repeated three times and averaged ($n = 3$). Data were given as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) method for comparing groups using the Stat View 4.5 software package (Abacus Concepts, Inc, Berkeley, CA). A significance level of $p < 0.05$ was adopted for all comparisons.

3. Results

3.1. Variations in phenolic compounds during in vitro digestion in the presence of Caco-2 cells

CAT, EC, B2 and B3 quantities at the end of each GSE digestion step are shown in Table 1. First we observed good stability of all compounds during saliva incubation. Although CAT and EC were stable during the gastric step, we noted a significant increase in B2 (9.3%) and B3 (24.9%) dimers at the end of incubation relative to the initial quantity. At the end of the 2 h intestinal incubation period on the apical side of Caco-2 cells grown in transwells, 56% of the initial CAT and 14.7% of the initial EC remained in the apical medium, but there were no dimers. However, no compounds could be detected in basal medium after the 2 h incubation period (data not shown). This was consistent with the absence of these compounds in Caco-2 cell monolayers.

The masking of studied compounds from GSE by proteins were investigated using acetonitrile extraction before HPLC analysis of the samples (Fig. 1(a)). When the same experiment as previously was performed with extraction, 59.8% and 52.2% of the initial CAT and EC quantities, respectively, were recovered in apical medium after 2 h of intestinal incubation, but no dimers were detected. Moreover, even after acetonitrile extraction, no compounds were detected in basal medium or in Caco-2 cell monolayers (data not shown).

3.2. Variations in phenolic compounds during in vitro digestion in the absence of Caco-2 cells

The stability of the studied compounds from GSE in the intestinal medium was investigated. After successive incubations of GSE in artificial saliva and gastric medium, this was subjected to intestinal medium in the absence of Caco-2 cells and acetonitrile extraction of the samples was performed to rule out the effect of interactions with proteins. Thus, after 2 h incubation in intestinal medium, 98.26%, 86.84%, 106.98% and 98.15% of the initial CAT, EC, B2 and B3 were recovered, respectively (Fig. 1(b)). However, when the same experiment was conducted without previous acetonitrile extraction, only 59.4%, 37.9%, 7.9% and 33.6% of CAT, EC, B2 and B3, respectively, remained at the end of intestinal step.

3.3. Effect of GSE on Caco-2 cell hydrolase activities

Exposure of Caco-2 cells for 2 h with predigested GSE at 0.3 g/l led to a significant decrease in hydrolase activities, particularly for alkaline phosphatase, with only 20.2% of control cell activity at the end of the incubation period. The activities of sucrase-isomaltase and aminopeptidase N in GSE-exposed cells represented 39.1% and 39.3% of control cells, respectively. Lastly, the intracellular and apical activities of LDH decreased in the presence of GSE, to reach 53.5% and 33.6% of control cell activities, respectively (Fig. 2).

4. Discussion

Salivary and gastric digestion of GSE thus did not affect CAT, EC and dimers, which appeared to be very stable through these incubations. These results corroborate those of Tsuchiya et al. (1997) as well as Spencer, Schroeter, Rechuer, and Rice-Evans (2001) and Spencer (2003), who documented the stability of green tea catechin and procyanidin oligomers, respectively, in saliva. In our study, no interactions seemed to occur between the investigated phenolic compounds and artificial saliva constituents, including casein and α-amylase. On the other hand, Arts et al. (2002) demonstrated interactions between β-casein and monomers, like CAT and EC, by way of proline residues (Luck et al., 1994). Nevertheless, the casein used herein was a blend of several caseins in proportions found in milk, with only 344 g/kg of β-casein (37% of total casein) (Cayot & Lorient, 1998) and a total proline ratio of only 10.14% of total amino acids versus 15.6% in β-casein alone. Moreover, GSE contains 67.4% (w/w) of procyanidins, but the studied monomers and dimers only represent 13% (w/w) of GSE. Around 54.4% (w/w) of procyanidins may thus contain compounds with higher degrees of polymerization, which are known to have better affinity for proteins than

Table 1
Recovery of CAT, EC, dimers B2 and B3 after each step of the in vitro digestion of 0.3 g/l GSE, consisting of successive incubations in saliva (10 min), gastric medium (60 min) and pancreatic medium (120 min) in presence of Caco-2 cells grown in transwell

% Recovery[a]										
	Saliva		Gastric medium		Intestinal medium					
					Apical medium		Cell monolayer		Basal medium	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CAT	98.6	0.9	98.3	1.0	56.1[b]	24.2	nd[c]	–	nd	–
EC	97.1	2.7	96.6	1.4	14.7[b]	6.6	nd	–	nd	–
B2	101.5	1.6	109.3[b]	1.3	nd	–	nd	–	nd	–
B3	102.4	1.1	124.9[b]	2.1	nd	–	nd	–	nd	–

[a] Percentage of initial quantities. Values are means ± SEM ($n = 3$). Data were analyzed by one-way ANOVA followed by the least significant difference test.
[b] Significantly different from initial sample.
[c] Not detected.

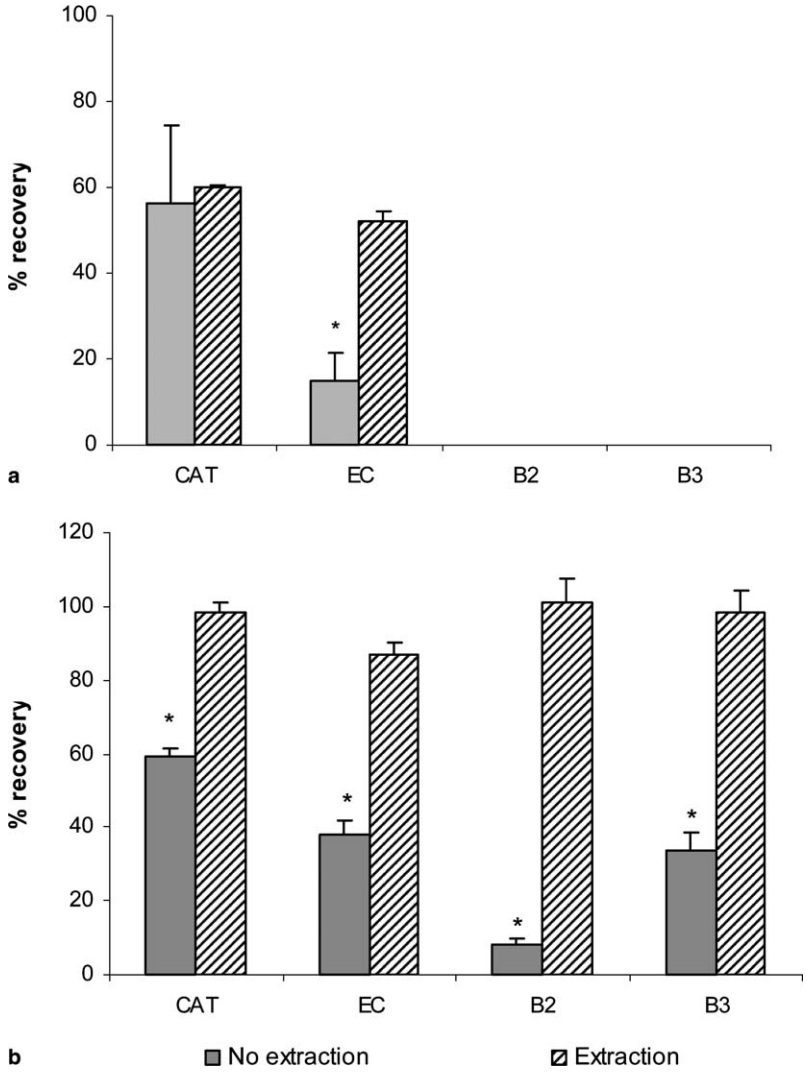

Fig. 1. Effect of acetonitrile extraction on phenolic compound recovery after in vitro digestion of 0.3 g/l GSE, consisting of successive incubations in saliva (10 min), gastric medium (60 min) and pancreatic medium (120 min). (a) Intestinal incubation was performed in the apical compartment of transwells in the presence of Caco-2 cells, (b) intestinal incubation was performed in tubes without Caco-2 cells. Phenolic compound recovery is expressed as a percentage of initial quantities and are means ± SEM ($n = 3$). For each compound, bars with asterisks are significantly different ($p < 0.05$).

monomers. de Freitas and Mateus (2001) have shown that low MW phenolic compounds seem to be too small to effectively crosslink globular proteins and demonstrated the low affinity of monomers and dimers for α-amylase. Moreover, the stability of flavan-3-ol monomers and dimers has previously been shown at acidic pH (Record & Lane, 2001; Spencer et al., 2000; Yoshino, Suzuki, Sasaki, Miyase, & Sano, 1999). However, it was documented that the decomposition of high-polymerized oligomers (>trimers) of procyanidins may occur at pH 2, mainly releasing dimers (Beart, Lilley, & Haslam, 1985). This could explain the slight increase in B2 and B3 dimers observed in our study through the gastric step. At the end of gastric digestion, CAT, EC and B2 and B3 dimers from GSE remained thus available for exposure to intestinal cells and possible absorption.

Regarding our results, only some of the initial monomers and no dimers remained in the apical medium after intestinal incubation of predigested GSE at 0.3 g/l in the presence of Caco-2 cells. In the study carried out without cell monolayer, all compounds remained totally present after 2 h of incubation in intestinal medium and after previous acetonitrile extraction, which suggests that these monomers and dimers are quite stable at pH 7. In contrast, according to Zhu et al. (2002), 20% of B2 dimers were degraded after 2 h of incubation at pH 7.4 and all dimers disappeared in intestinal juice at pH 8.5. It was also shown that EC is less stable than CAT at this pH since, according to the authors, about 15 to 34% are degraded at pH 7.5 within 2 h (Record & Lane, 2001; Yoshino et al., 1999; Zhu et al., 2002). All of the studied compounds were thus stable at pH 7 during intestinal digestion in the absence of Caco-2 cells but when the same experiment was conducted without acetonitrile extraction before HPLC analysis, very low quantities of compounds remained after intestinal incubation, suggesting that interactions could

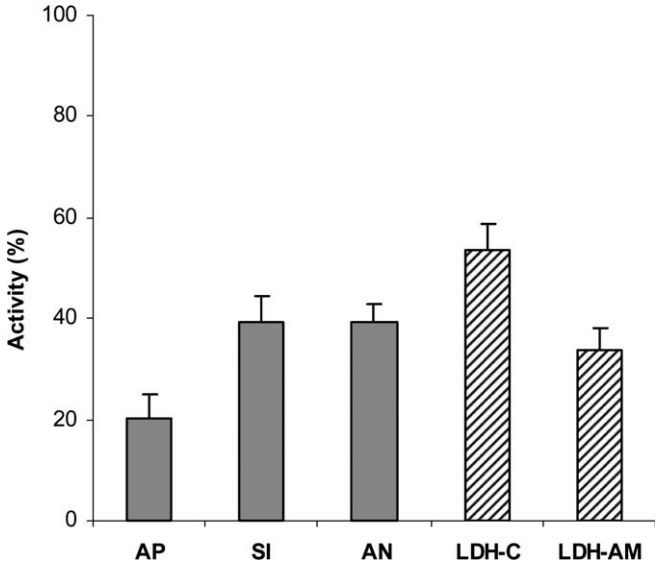

Fig. 2. Activity of Caco-2 cell hydrolases, at the end of in vitro digestion of 0.3 g/l GSE, consisting of successive incubations in saliva (10 min), gastric medium (60 min) and pancreatic medium in the presence of Caco-2 cells (120 min). Black bars: brush border enzymes, i.e., alkaline phosphatase (AP), sucrase-isomaltase (SI) and aminopeptidase N (AN); white bars: cytoplasmic enzyme, lactate dehydrogenase in cells (LDH-C) and in apical medium (LDH-AM). Activities are expressed as a percentage of control cell activities and are means ± SEM ($n = 9$).

occur between proteins and these compounds, particularly the B2 dimer. Indeed, the intestinal medium used was a protein-rich solution containing pancreatic enzymes. Although some authors have shown the possible interaction of pancreatic α-amylase and trypsin with phenolic compounds (Rhon et al., 2002), other showed that CAT was not able to bind trypsin (Fickel, Pitra, Joest, & Hofmann, 1999). The results of the present study suggest that interactions may occur between digestive proteins and monomers and dimers of flavanols from GSE during pancreatic digestion.

Otherwise, a part of EC and all dimers have disappeared at the end of the intestinal digestion in the presence of Caco-2 cells, suggesting that EC could partly interact with proteins located around brush border Caco-2 cells, since acetonitrile extraction unmasked a significant portion of this monomer by breaking interactions with proteins. However, the total disappearance of dimers could not be explained by interactions since acetonitrile extraction had no effect on their recovery. The presence of direct interactions was confirmed with the clear decrease in Caco-2 cells enzymatic activities after contact with predigested GSE. However, very few studies showed the possibility of such interactions. Welsch et al. (1989) reported in vitro inhibition of sucrase activity by some phenolic compounds and particularly by unoxidized and oxidized EC, with a 45% and 38% decrease in activity, respectively. Tebib et al. (1995) assessed the in vivo and in vitro activity of alkaline phosphatase and sucrase after exposure to a grape seed tannin extract (1.5 g/l) and reported a respective decrease of 63 and 37% in vivo (over a 31-day period) and about 50% and 75% in vitro (over a 30-min period). Nevertheless, it should be noted that high-polymerized oligomers are more able to bind proteins than monomers (Sarni-Manchado et al., 1999) and could be responsible for the enzymatic activity decreases observed in our study. Concerning intracellular LDH, the presence of GSE led to a weak inhibition of activity, suggesting that some compounds could have passed through the cell membrane and penetrated into the cytoplasm.

Despite a few interactions between phenolic compounds and proteins, some compounds could have been partly absorbed by Caco-2 cells, since previous in vivo studies highlighted partial absorption of procyanidins and flavan-3-ols, as demonstrated by the presence of these compounds in plasma or urine (Baba et al., 2001a, 2001b; Baba, Osakabe, Natsume, & Terao, 2002; Bell et al., 2000; for review see Manach et al., 2005). However, in the present study, no phenolic compounds were detected in their native form in basal medium, which rules out the possibility of paracellular transport of such compounds. These results did not corroborate those of Deprez et al. (2001), who suggested that CAT and procyanidin dimers may be transported by Caco-2 cells through a paracellular route. Nevertheless, for their study, they used higher CAT doses (1 mM) than those used in our study. Thus, these compounds could not be able to penetrate the membrane or could be metabolized by cells. Indeed, according to some in vivo and in vitro studies, flavanols and procyanidins may be metabolized in intestinal cells into glucuronide/methylated conjugates (Donovan et al., 2001; Kuhnle et al., 2000; Natsume et al., 2003; for review see Spencer, 2003). Otherwise, it was previously demonstrated that Caco-2 cells can metabolize certain phenolic compounds (Galijatovic et al., 1999; Murota et al., 2002; Vaidyanathan & Walle, 2001, 2003; Walle, Galijatovic, & Walle, 1999), and some intestinal enzymes such as UDP-glucuronosyltransferase or phenolsulfotransferase were also found to be present in this cell line (Boulenc, 1997; Hillgren et al., 1995). While Deprez et al. (2001) did not detect any metabolites after 4 h of incubation of 1 mM catechin on Caco-2 cells, Vaidyanathan and Walle (2001) have shown sulfatation of epicatechin by Caco-2 cells after 3 h of EC incubation (5–50 μM), associated with the absence of apical to basal transport and cell accumulation of native EC. In addition, these authors highlighted the presence of excretion of conjugated EC metabolites from basal to apical medium by the multi-drug resistance protein (MRP2). Moreover, efflux from Caco-2 cells, mediated by MRP2, has also been shown for other phenolic compounds such as chrysin (Galijatovic et al., 1999; Walle et al., 1999), quercetin (Walgren, Walle, & Walle, 1998), (−)-epicatechin-3-gallate (Vaidyanathan & Walle, 2001) and green tea catechins (Zhang, Zheng, Chow, & Zuo, 2004). Concerning procyanidin dimers, it appears that the extent of their polymerisation could have a negative effect on their intestinal absorption (Gonthier et al., 2003). However, according to the authors, after consumption of cocoa-derived procyanidin dimers, such as B2 (epicate-

chin-(4β-8)-epicatechin) or B5 (epicatechin-(4β-6)-epicatechin), only epicatechin is detected after transfer across the small intestine, thus suggesting degradation of dimers into monomer epicatechin (Holt et al., 2002; Spencer et al., 2001). On the other hand, Sano, Yamakoshi, Tokutake, Kubota, and Kikuchi (2003) showed the presence of B1 dimers (epicatechin-(4β-8)-catechin) at 10.6 nM concentration in human serum 2 h after consumption of 2 g of proanthocyanidin-rich grape seed extract.

Thus, when GSE was subjected to in vitro digestion associated with Caco-2 cells, the absorption and metabolism of the studied compounds were still possible, so further studies are needed to investigate the presence of conjugates in basal and apical medium as well as in Caco-2 cells.

Nevertheless, it should be taken into account that the loss of phenolic compounds in apical medium after digestion in the presence of Caco-2 cells could be partly explained by the possible oxidation of compounds in the cell culture medium. In previous studies, it was shown that the addition of phenolic compounds in the cell culture medium led to the generation of substantial amounts of H_2O_2 (Cambon Roques et al., 2002; Long, Clement, & Halliwell, 2000). In a study performed in our laboratory, inorganic salts were found to be the major culprits (Cambon Roques et al., 2002), particularly the Ca^{2+} cation . When GSE (0.4 g/l) was incubated in culture medium at 37 °C, about 40% of CAT and EC were lost within 24 h as compared to only 15% in culture medium without Ca^{2+} (data not published).

We thus obtained evidence, when using an in vitro digestion system, that only pancreatic digestion in the presence of Caco-2 cells plays a determining role in the availability of phenolic compounds, since salivary and gastric digestions had no effect on their stability. This is the first study that has been carried out using in vitro digestion with artificial saliva, and further studies are now needed to compare its efficacity with human saliva. In this study, which was focused on protein–phenolic compound interactions, we showed that the main interactions occurred during the intestinal step with digestive juice proteins and/or brush border cell proteins such as enzymes. Although absorption might have occurred, no compounds were detected in their native form in the basal compartment and further investigations are needed to reveal the presence of possible conjugated forms. Therefore, additional work focused on the bioavailability of phenolic compounds and using the Caco-2 cell model is necessary to study these interactions in the presence of a simulated complete meal.

Acknowledgments

The authors gratefully acknowledge Mr. Eric Fesquet and Mrs. Nelly Urban (La Gardonnenque S.A., 30360, Cruviers-Lascours, France) for the preparation and gift of the grape seed extract.

References

Aherne, S. A., & O'Brien, N. M. (2002). Dietary flavonols: chemistry, food content, and metabolism. *Nutrition, 18*, 75–81.

Arts, I. C. W., Hollman, P. C., & Kromhout, D. (1999). Chocolate as a source of tea flavonoids. *Lancet, 354*, 488.

Arts, I. C. W., Van de Putte, B., & Hollman, P. C. H. (2000). Catechin contents of foods commonly consumed in the Netherlands 2. Tea, wine, fruit juices and chocolate milk. *Journal of Agricultural and Food Chemistry, 48*, 1752–1757.

Arts, M. J. T. J., Haenen, G. R. M. M., Wilms, L. C., Beestra, S. A. J. N., Heijnen, C. G. M, Voss, H. P., et al. (2002). Interactions between flavonoids and proteins: effect on the total antioxydant capacity. *Journal of Agricultural and Food Chemistry, 50*, 1184–1187.

Baba, S., Osakabe, N., Natsume, M., Muto, Y., Takizawa, T., & Terao, J. (2001a). In vivo comparison of the bioavailability of (+)-catechin, (−)-epicatechin and their mixture in orally administered rats. *Journal of Nutrition, 13*, 2885–2891.

Baba, S., Osakabe, N., Natsume, M., Muto, Y., Takizawa, T., & Terao, J. (2001b). Absorption and urinary excretion of (−)-epicatechin after administration of different levels of cocoa powder or (−)-epicatechin in rats. *Journal of Agricultural and Food Chemistry, 49*, 6050–6056.

Baba, S., Osakabe, N., Natsume, M., & Terao, J. (2002). Absorption and urinary excretion B2 [epicatechin-(4β-8)-epicatechin] in rats of procyanidin. *Free Radical Biology & Medicine, 33*, 142–148.

Bagchi, D., Sen, C. K., Ray, S. D., Das, D. K., Bagchi, M., Preuss, H. G., et al. (2003). Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutation Research, 523–524*, 87–97.

Beart, J. E., Lilley, T. H., & Haslam, E. (1985). Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalized decomposition; observations on some polymeric proanthocyanidins. *Journal of the Chemical Society – Perkin Transactions II*, 1439–1443.

Bell, J. R. C., Donovan, J. L., Wong, R., Waterhouse, A. L., German, J. B., Walzem, R. L., et al. (2000). (+)-Catechin in human plasma after ingestion of a single serving of reconstituted red wine. *American Journal of Clinical Nutrition, 71*, 103–108.

Bessey, O. A., Lowry, O. H., & Brock, M. J. (1946). Rapid colorimetric method for the determination of alkaline phosphatase in five cubic millimeters of serum. *Journal of Biological Chemistry, 164*, 321–329.

Biganzoli, E., Cavenaghi, L. A., Rossi, R., Brunati, M. C., & Nolli, M. L. (1999). Use of a Caco-2 cell culture model for the characterization of intestinal absorption of antibiotics. *Il Farmaco, 54*, 59–599.

Boulenc, X. (1997). Intestinal cell models. Their use in evaluating the metabolism and absorption of xenobiotics. *STP Pharma Sciences, 7*, 259–269.

Burda, S., & Oleszek, W. (2001). Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry, 49*, 2774–2779.

Cambon Roques, S., Landrault, N., Teissèdre, P.-L., Laurent, C., Besançon, P., Rouanet, J.-M., et al. (2002). Hydrogen peroxide generation in Caco-2 cell culture medium by addition of phenolic compounds: effect of ascorbic acid. *Free Radical Research, 36*, 593–599.

Carando, S., Teissèdre, P.-L., Pascual-martinez, L., & Cabanis, J.-C. (1999). Levels of flavan-3-ols in french wines. *Journal of Agricultural and Food Chemistry, 47*, 4161–4166.

Cayot, P., & Lorient, D. (1998). *Structures et technofonctions des protéines du lait*. Paris: Tec et Doc, Lavoisier.

Cook, N. C., & Sammam, S. (1996). Flavonoids – chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry, 7*, 66–76.

Deprez, S., Mila, I., Huneau, J. F., Tome, D., & Scalbert, A. (2001). Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial caco-2 cells. *Antioxidants & Redox Signaling, 3*, 957–967.

Donovan, J. L., Crespy, V., Manach, C., Morand, C., Besson, C., Scalbert, A., et al. (2001). Catechin is metabolized by both the small intestine and liver of rats. *Journal of Nutrition, 131*, 1753–1757.

Ekmekioglu, C., Ekmekioglu, A., & Marktl, W. (2000). Magnesium transport from aqueous solutions across Caco-2 cells – an experimental model for intestinal bioavailability studies. Physiological considerations and recommendations. *Magnesium Research, 13*, 93–102.

Fickel, J., Pitra, C., Joest, B. A., & Hofmann, R. R. (1999). A novel method to evaluate the relative tannin-binding capacities of salivary proteins. *Comparative Biochemistry and Physiology. Part C, 122*, 225–229.

de Freitas, V., & Mateus, N. (2001). Structural features of procyanidins interactions with salivary proteins. *Journal of Agricultural and Food Chemistry, 49*, 940–945.

Fuleki, T., & Ricardo da Silva, J. M. (1997). Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. *Journal of Agricultural and Food Chemistry, 45*, 1156–1160.

Galijatovic, A., Otake, Y., Walle, U. K., & Walle, T. (1999). Extensive metabolism of the flavonoid chrysin by human Caco-2 and Hep-G2 cells. *Xenobiotica, 29*, 1241–1256.

Glahn, R. P., Wien, E. M., Van Campen, D. R., & Miller, D. D. (1996). Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: use of a novel in vitro method for rapid estimation of iron bioavailability. *Journal of Nutrition, 126*, 332–339.

Glahn, R. P., Cheng, Z., & Welch, R. M. (2002). Comparison of iron bioavailibility from 15 rice genotypes: studies using an in vitro digestion/caco-2 cell culture model. *Journal of Agricultural and Food Chemistry, 50*, 3586–3591.

Gonthier, M.-P., Donovan, J. L., Texier, O., Felgines, C., Rémésy, C., & Scalbert, A. (2003). Metabolism of dietary procyanidins in rats. *Free Radical Biology & Medicine, 35*, 837–844.

Hammerstone, J. F., Lazarus, S. A., & Schmitz, H. H. (2000). Procyanidin content and variation in some commonly consumed foods. *Journal of Nutrition, 130*, 2086S–2092S.

Hidalgo, I. J., Raub, T. J., & Borchard, R. T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology, 96*, 736–749.

Hillgren, K. M., Kato, A., & Borchardt, R. T. (1995). In vitro systems for studying intestinal drug absorption. *Medicinal Research Reviews, 15*, 83–109.

Holt, R. R., Lazarus, S. A., Sullards, M. C., Zhu, Q. Y., Schramm, D. D., Hammerstone, J. F., et al. (2002). Procyanidin dimer B2 (epicatechin-(4β-8)-epicatechin) in human plasma after the consumption of a flavanol-rich cocoa. *American Journal of Clinical Nutrition, 76*, 798–804.

Jezyk, N., Li, C., Stewart, B. H., Wu, X., Bockbrader, H. N., & Fleisher, D. (1999). Transport of pregabalin in rat intestine and Caco-2 monolayers. *Pharmaceutical Research, 16*, 519–526.

Khokhar, S., & Magnusdottir, S. G. M. (2002). Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *Journal of Agricultural and Food Chemistry, 50*, 565–570.

Kuhnle, G., Spencer, J. P. E., Schroeter, H., Shenoy, B., Debnam, E. S., Srai, K. S., et al. (2000). Epicatechin and catechin are *O*-methylated and glucuronidated in the small intestine. *Biochemical and Biophysical Research Communications, 277*, 507–512.

Lameula-Raventos, R. M., & Waterhouse, A. L. (1994). A direct HPLC separation of wine phenolics. *American Journal of Enology and Viticulture, 45*, 1–5.

Landrault, N., Poucheret, P., Ravel, P., Gasc, F., Cros, G., & Teissèdre, P.-L. (2001). Antioxidant capacities and phenolics levels of french wines from different varieties and vintages. *Journal of Agricultural and Food Chemistry, 49*, 3341–3348.

Lee, M.-J., Prabhu, S., Meng, X., Li, C., & Yang, C. S. (2000). A improved method for the determination of green and black tea polyphenols in biomatrices by high-performance liquid chromatography with coulometric array detection. *Analytical Biochemistry, 279*, 164–169.

Liu, Y., & Hu, M. (2002). Absorption and metabolism of flavonoids in the Caco-2 cell culture model and a perfused rat intestinal model. *Drug Metabolism and Disposition, 30*, 370–377.

Long, L. H., Clement, M. V., & Halliwell, B. (2000). Artifacts in cell culture: rapid generation of hydrogen peroxyde on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochemical and Biophysical Research Communications, 273*, 50–53.

Lu, Y., & Bennick, A. (1998). Interaction of tannin with human salivary proline-rich proteins. *Archives of Oral Biology, 43*, 717–728.

Luck, G., Liao, H., Murray, N. J., Grimmer, H. R., Warminski, E. E., Williamson, M. P., et al. (1994). Polyphenols, astringency and proline-rich proteins. *Phytochemistry, 37*, 357–371.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition, 81*, 230S–242S.

Maroux, S., Louvard, D., & Baratti, J. (1973). The aminopeptidase from hog intestinal brush border. *Biochimica and Biophysica Acta, 32*, 282–295.

Messer, M., & Dahlqvist, A. (1966). A one-step ultramicro method for the assay of intestinal disaccharidases. *Analytical Biochemistry, 14*, 376–392.

Murota, K., Shimizu, S., Miyamoto, S., Izumi, T., Obata, A., Kikuchi, M., et al. (2002). Unique uptake and transport of isoflavone aglycones by human intestinal Caco-2 cells: comparison of isoflavonoids and flavonoids. *Journal of Nutrition, 132*, 1956–1961.

Natsume, M., Osakabe, N., Oyama, M., Sasaki, M., Baba, S., Nakamura, Y., et al. (2003). Structures of (−)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (−)-epicatechin: differences between human and rat. *Free Radical Biology & Medicine, 34*, 840–849.

Planes, P., Rouanet, J.-M., Laurent, C., Baccou, J.-C., Besançon, P., & Caporiccio, B. (2002). Magnesium bioavailability from magnesium-fortified spirulina in cultured human intestinal Caco-2 cells. *Food Chemistry, 77*, 213–218.

Puyfoulhoux, G., Rouanet, J.-M., Besançon, P., Baroux, B., Baccou, J.-C., & Caporiccio, B. (2001). Iron availability from iron-fortified spirulina by an in vitro digestion/Caco-2 cell culture model. *Journal of Agricultural and Food Chemistry, 49*, 1625–1629.

Record, I. R., & Lane, J. M. (2001). Simulated intestinal digestion of green and black teas. *Food Chemistry, 73*, 481–486.

Rhon, S., Rawel, H. M., & Kroll, J. (2002). Inhibitory effects of plant phenols on the activity of selected enzymes. *Journal of Agricultural and Food Chemistry, 50*, 3566–3571.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine, 20*, 933–956.

Sano, A., Yamakoshi, J., Tokutake, S., Kubota, T. K., & Kikuchi, M. (2003). Procyandin B1 is detected in human serum after intake of proanthocyanidins-rich grape ssed extract. *Bioscience Biotechnology and Biochemistry, 67*, 1140–1143.

Sarni-Manchado, P., Cheynier, V., & Moutounet, M. (1999). Interactions of grape seed tannins with salivary proteins. *Journal of Agricultural and Food Chemistry, 47*, 42–47.

Smith, S. K., Krohn, R. I., Mallia, A. K., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*, 76–85.

Spencer, J. P. E., Chaudry, F., Pannala, A. S., Srai, S. K., Debnam, E., & Rice-Evans, C. (2000). Decomposition of cocoa procyanidins in the gastric milieu. *Biochemical and Biophysical Research Communications, 272*, 236–241.

Spencer, J. P. E., Schroeter, H., Rechuer, A., & Rice-Evans, C. (2001). Incubation of procyanidins oligomers (dimers to hexamers) in human saliva for up to 30 min does not result in modification of these compounds or their decomposition into smaller oligomeric unit, supporting that these compounds remain intact in the mouth before entering the stomach. *Antioxidants & Redox Signaling, 3*, 1023–1040.

Spencer, J. P. E. (2003). Metabolism of tea flavonoids in the gastrointestinal tract. *Journal of Nutrition, 133*, 3255S–3261S.

Tebib, K., Rouanet, J.-M., & Besançon, P. (1995). Effect of grape seed tannins on the activity of some rat intestinal enzyme activities. *Enzyme Protein, 48*, 51–60.

Tsuchiya, H., Sato, M., Kato, H., Okubo, T., Juneja, L. R., & Kim, M. (1997). Simultaneous determination of catechins in human saliva by high-performance liquid chromatography (−)-epicatechin by the human intestinal cell line Caco-2. *Journal of Chromatography B, 703*, 253–258.

Vaidyanathan, J. B., & Walle, T. (2001). Transport and metabolism of the tea flavonoid (−)-epicatechin by the human intestinal cell line Caco-2. *Pharmaceutical Research, 18*, 1420–1425.

Vaidyanathan, J. B., & Walle, T. (2003). Cellular uptake and efflux of the tea flavonoid (−)-epicatechin-3-gallate in the human intestinal cell line Caco-2. *The Journal of Pharmacology and Experimental Therapeutics, 307*, 745–752.

Walgren, R. A., Walle, U. K., & Walle, T. (1998). Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochemical Pharmacology, 55*, 1721–1727.

Walle, U. K., Galijatovic, A., & Walle, T. (1999). Transport of the flavonoid chrysin and its conjugated metabolites by the human intestinal cell line Caco-2. *Biochemical Pharmacology, 58*, 431–438.

Waterhouse, A. L., & Teissèdre, P.-L. (1997). Levels of phenolics in California varietal wines. In T. C. Watkins (Ed.), *Wine nutritional and therapeutic benefits. ACS symposium series* (Vol. 661, pp. 12–23). Washington, DC: American Chemical Society.

Welsch, C. A., Lachance, P. A., & Wasserman, B. P. (1989). Effects of native and oxydized phenolic compounds on sucrase activity in rat brush border membrane vesicles. *Journal of Nutrition, 119*, 1737–1740.

Williamson, G., & Manach, C. (2005). Bioavailability and bioefficacy of polyphenols in humans II. Review of 93 intervention studies. *American Journal of Clinical Nutrition, 81*, 243S–255S.

Wroblewski, K., Muhandiram, R., Chakrabartty, A., & Bennick, A. (2001). The molecular interaction of human salivary histatins with polyphenolic compounds. *European Journal of Biochemistry, 268*, 4384–4397.

Yoshino, K., Suzuki, M., Sasaki, K., Miyase, T., & Sano, M. (1999). Formation of antioxydants from (−)-epigallocatechin gallate in mild alkaline fluids, such as authentic intestinal juice and mouse plasma. *The Journal of Nutritional Biochemistry, 10*, 223–229.

Zhang, L., Zheng, Y., Chow, M. S. S., & Zuo, Z. (2004). Investigation of intestinal absorption and disposition of green tea catechins by caco-2 monolayer model. *International Journal of Pharmaceutics, 287*, 1–12.

Zhu, Q. Y., Holt, R. R., Lazarus, S. A., Ensunsa, J. L., Hammerstone, J. F., Schmitz, H. H., et al. (2002). Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. *Journal of Agricultural and Food Chemistry, 50*, 1700–1705.