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Analysis of catechins from milk–tea beverages by enzyme assisted extraction followed by high performance liquid chromatography

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Abstract

Extraction and analysis of physiologically significant tea catechins from complex food matrices is complicated by strong association of tea catechins with macronutrients such as proteins. Dependable extraction methods are required to accurately assess and validate levels of bioactive tea catechins in new products. The objective of this work was to investigate recovery of tea catechins from dairy matrices and evaluate pepsin treatment as an enzymatic step to enhance catechin recovery from milk and other protein rich formulations. Brewed green tea was combined with skim milk to produce test solutions ranging from 10% to 50% milk. Samples were treated by either acid (0.1 N HCl), methanol, or by pepsin (40.0 mg/mL). Following treatments, samples were centrifuged and supernatants analyzed for tea catechins by reversed phase C18 HPLC with photodiode array detection. Recovery of total catechins was highest for pepsin treated samples (89–102%), followed by methanol deproteination (78–87%) and acid precipitation (20–74%) with values decreasing with increased milk content. Individual recovery of gallated catechins, namely epigallocatechin-gallate (EGCG) and epicatechin-gallate (ECG), was most affected by the presence and level of milk. The usefulness of pepsin treatment for enhancing recovery of tea catechins was further demonstrated in analysis of commercial soy and milk–tea beverages.

Keywords: Tea; Catechins; Flavonoids; Milk; Extraction; Enzyme treatment; Pepsin; HPLC

1. Introduction

Tea (*Camellia sinensis*) is the second most consumed beverage in the world after water. Epidemiological studies have associated numerous health benefits with tea consumption including prevention of chronic disease such as cancer and cardiovascular disorders (Borrelli, Capasso, Russo, & Ernst, 2004; Geleijnse, Launer, van der Kuip, Hofman, & Witteman, 2002; Hollman, Feskens, & Katan, 1999; Mukamal, Maclure, Muller, Sherwood, & Mittleman, 2002; Scalbert, Manach, Morand, Remesy, & Jimenez, 2005; Sesso, Gaziano, Buring, & Hennekens, 1999; Yang, Lu, Wu, Wu, & Chang, 2004). Interest in tea polyphenols as potential physiologically active agents has intensified due to the diversity and high concentration of these compounds found in brewed tea. Catechins represent approximately 80% of the total flavonoid content of green tea. Major green tea catechins include epicatechin (EC), epigallocatechin (EGC), epigallocatechin-gallate (EGCG), and epicatechin-gallate (ECG), (Fig. 1). Health benefits of tea catechins are often associated to their antioxidant activities including scavenging of reactive oxygen and nitrogen species, free metal chelation, inhibition of transcriptional factors such as Activator Protein 1, and inhibition of oxidative enzymes such as lipoxygenase and cycloxygenase (reviewed in Higdon & Frei, 2003).

Abbreviations: HPLC, high performance liquid chromatography; UV–Vis, ultraviolet and visible; EC, epicatechin; ECG, epicatechin-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-gallate.

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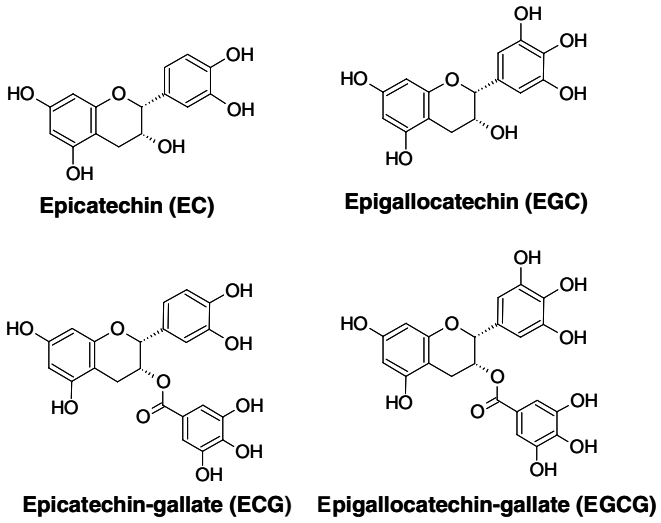

Fig. 1. Structure of major catechins from green tea and corresponding infusions.

With epidemiological and biological data supporting a potential protective role for tea and tea catechins, development of new products with tea as active ingredients has expanded to include ready-to-drink (RTD) tea beverages, cereal bars, ice creams, confections and pet foods. In many countries brewed and RTD teas often are combined or formulated with milk as a common adjunct to improve sensory properties. This is especially true in traditional consumption of black tea. While green tea has not traditionally been consumed with milk, new trends and RTD green tea products formulated on soy and dairy bases are growing in popularity. However, as tea product matrix complexity increases, accurate and reliable measurement of physiologically significant tea catechins becomes more challenging.

Qualitative and quantitative assessment of tea catechins is critical to development and clinical assessment of new products ensuring accurate dosing and assessment of catechin stability to processing and storage conditions. Currently, a number of methods exist for simple matrices such as tea leaf or infusions (Chandra & Gonzalez de Mejia, 2004; Henning et al., 2003; Lee & Ong, 2000; Manning & Roberts, 2003; Yao et al., 2004). Analysis is commonly performed by hot water or solvent extraction from the tea matrix followed by direct injection onto a reverse phase high performance liquid chromatography (RP-HPLC) system with ultraviolet and visible (UV–Vis), or electrochemical (EC) detection. Efforts have expanded analysis of common catechins to include fruit and vegetable matrices (Arts & Hollman, 1998; Kammerer, Claus, Carle, & Schieber, 2004; Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003). While extremely effective for tea leaf or leaf infusions, and select fruit and vegetables; these methods are not suitable for effective extraction recovery of catechins in complex food formulations such as milk based products.

Milk based products represent a very complex matrix where strong catechin–protein interactions are well known to occur (Siebert, Troukhanover, & Lynn, 1996) and can directly interfere with accurate catechin determination by significantly reducing analytical recovery. Presence of proteins in a sample matrix requires a deproteination step prior to analysis in order to avoid precipitation onto the HPLC column during analysis. Alternative methods to improve recovery and minimize chromatographic complexity involve solid phase extraction (SPE) procedures for sample clean-up of complex biological samples (Chu et al., 2004; Lee, Prabhu, Meng, Li, & Yang, 2001). While effective at removing interferences, these methods can be expensive and do introduce variability and artifacts by increasing sample manipulation. Furthermore, SPE methods rely on primary extraction techniques and thus may not address the need to disrupt protein–catechin interactions affecting recovery. Analysis of tea catechins from complex protein rich food matrices requires development of improved methodology maximizing recovery of catechins while minimizing variability of analysis.

The present study describes application of short-time pepsin treatment in an enzymatic assisted extraction for release of tea catechins from milk and soy matrices prior to analysis by HPLC with photodiode array (PDA) detection. Experiments involving a range of milk–tea concentrations were performed to determine impact of milk level on catechin analytical recovery. Assessment of commercially available milk–tea and soymilk–tea products was conducted to illustrate the usefulness of this method in analysis of commercial RTD tea beverages.

2. Materials and methods

2.1. Chemicals and standards

HPLC solvents, methanol, acetonitrile and glacial acetic acid (Mallinckrodt-Baker, Phillipsburg, NJ) were of certified HPLC and ACS grade. Porcine pepsin utilized in enzyme assisted extraction techniques was purchased from Sigma Aldrich (St. Louis, MO). The following standards were obtained: catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin-gallate (EGCG), epicatechin-gallate (ECG), and caffeine (CAF) (Sigma Aldrich, St. Louis, MO). Each standard was dissolved in water/acetic acid (98:2, v/v) and filtered through a 0.45 μm PTFE filter prior to injection.

2.2. Sample preparation

Skim milk and Premium Green Tea (The Stash Company; Portland, OR) were purchased at a local market. Samples of commercial ready to drink (RTD) green tea–soymilk (Pearl Organic Soymilk Green Tea, Kikkoman) and black tea–milk (Chai Tea Latte, Oregon Chai) beverages were purchased from a local market. Tea infusions were prepared by extracting 2.2 g of green tea leaf in

250 mL of boiling water (100 °C) for 5 min with mild agitation. After brewing, tea infusions were filtered through fluted filter paper to remove any tea leaf particles. Tea–milk preparations were produced by combining brewed tea with skim milk to levels of 10%, 20% and 50% milk (v/v) and allowed to equilibrate for 20 min at 4 °C. A parallel set of tea–water preparations were produced by dilution with double distilled water in to order assess the impact of milk on catechin recovery. All preparations were allowed to equilibrate to room temperature prior to further sampling, extraction and analysis.

2.3. Catechin extractions

Catechin extraction was initiated by deproteination with either acid, methanol, or by pepsin for enzyme assisted extraction. For *acid precipitation*, 2 mL aliquots of tea sample were treated with 6 mL of 0.1 N HCl for 1 min under vigorous mixing. *Solvent extraction* was accomplished by addition of 6 mL of methanol to a 2 mL aliquot of tea sample followed by 1 min of vigorous mixing. *Enzyme assisted extraction* of tea samples was initiated by addition of 6 mL of pepsin (40 mg/mL in 0.1 N HCl) to a 2 mL aliquot of tea preparation. The mixture was allowed to react from 15 min at 37 °C with gentle mixing. Both tea–milk and tea–water samples were treated in the same manner. Aliquots of each treatment were combined with 2% aqueous acetic acid 1:1 v/v and centrifuged at 14,000*g* for 5 min. Supernatants were collected and filtered through a 0.45 μm PTFE filter and analyzed immediately by HPLC.

2.4. Catechin analysis

Analysis of catechins was performed by use of a Waters model 2695 HPLC system equipped with a model 2996 photodiode array detector. A Waters NovaPak C18 (3.8 mm i.d. × 150 mm) reversed phase (RP) column (Milford, MA) with a guard column also packed with Waters NovaPak C18. Catechins were separated by gradient elution with a flow rate of 1.0 mL/min at 35 °C using a binary mobile phase of water/acetic acid (98:2, v/v) in reservoir A and acetonitrile in reservoir B. Initial conditions were solvent proportions of 99:1 A/B with a linear gradient to 70:30 A/B over 20 min followed by a 5 min linear gradient back to 99:1 A/B and 5 min equilibration at initial conditions for a total chromatographic run time of 30 min. Detection and tentative identification of major tea catechins was accomplished using in-line PDA data between 200 and 500 nm. Calibrations plots for quantification were constructed from injection of authentic standards of CAF, EC, EGC, EGCG, and ECG.

2.5. Data analysis

Percent recovery for each catechin derivative from tea–milk preparations was determined by calculation of the percent of individual catechin levels in tea–milk preparations relative to levels in tea–water preparations. All data were analyzed using SAS 9.1.3 (SAS Institute, Cary, NC). Descriptive statistics including mean and standard error of mean (SEM) were calculated for each catechin derivative's extraction recovery and absolute content. Group differences were determined by analysis of variance with Fischer's least significant difference (LSD) post hoc test ($\alpha < 0.05$).

3. Results and discussion

3.1. HPLC analysis

Separation of major catechins from a green tea infusion by a RPC18 HPLC can be seen in Fig. 2. Major catechins and caffeine were tentatively identified based on co-chromatography and comparison of their electronic absorption spectra with that of authentic standards. Total catechin content was determined to be 71.1 mg/100 mL. EGCG (33.4 mg/100 mL) was the major catechin component followed by EGC (19.2 mg/100 mL), ECG (11.2 mg/100 mL) and EC (7.3 mg/100 mL). Caffeine amounts were determined to be 27.1 mg/100 mL. These results are similar to those previously described for green tea (Henning et al.,

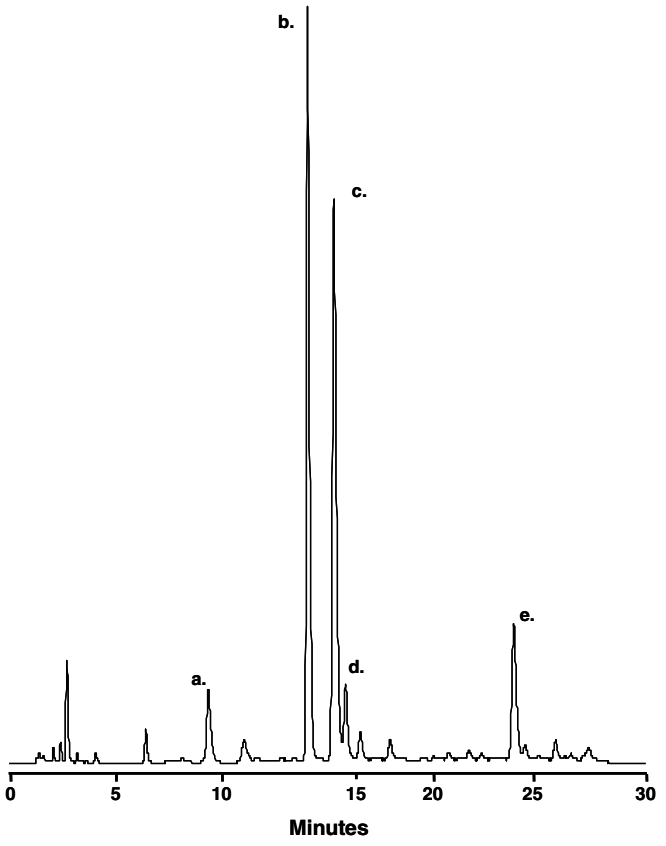

Fig. 2. HPLC separation of major green tea catechins. Peak identifications: (a) EGC, (b) Caffeine, (c) EGCG, (d) EC, and (e) ECG. Online UV–Vis spectra were collected from 200 to 500 nm. Response at 280 nm shown.

2003). Other minor tea catechins and phenolic components including catechin, gallocatechin-gallate, catechin gallate and gallocatechin were detected but not quantified in our efforts as they represented only a minor portion of the total catechin fraction.

3.2. Effect of milk on catechin recovery

Influence of milk on recovery of catechins was examined in test preparations of milk and green tea. Green tea infusions were combined with skim milk to produce 0%, 10%, 20% and 50% solutions of skim milk in tea. Following acid precipitation and centrifugation, supernatants were recovered and catechin content determined by HPLC. Recovery of tea catechins was markedly affected by milk content with an inverse association demonstrated by decreased catechin recovery with increased milk content (Fig. 3). Relative to milk-free control samples recovery of total catechins was determined to be 70%, 46%, and 34% for 10%, 20% and 50% milk content, respectively (Fig. 3(c)). Gallated catechins EGCG and ECG were most affected by milk addition with 70–80% loss at 50% milk concentrations (Fig. 3(b)). Similar concentration dependent loss of gallated tea catechins in milk was noted previously (Catterall, Kassimi, Clifford, & Ioannides, 2003). However, contrary to these previous reports, we observed a significant decline in the non-gallated catechins EC and EGC as well as caffeine (Figs. 3(a) and (c)). Based on these data 20% and 50% milk contents were chosen for further work on comparison of extraction methods.

Strong protein–flavonoid interactions are known to exist and likely play a significant role in the poor recovery of tea catechins from protein rich matrices. Strong hydrophobic association between galloyl rings on monomer and polymer polyphenols are known to associate with proline rich peptides fragments and are further stabilized by secondary hydrogen-bonding effects (Baxter, Lilley, Haslam, & Williamson, 1997; Murray, Williamson, Lilley, & Haslam, 1994). Interaction between catechins and specific milk proteins has been studied including α-casein, β-casein, κ-casein and albumin (Arts et al., 2002; Jöbstl, O'Connell, Fairclough, & Williamson, 2004; Papadopoulou, Green, & Frazier, 2005). In general these studies conclude that ionic, hydrophobic and hydrogen bonding forces are all important factors in catechin–protein interaction. In addition, it is also possible that a number of calcium–catechin interactions and caffeine–catechin interactions further contributed to the poor recovery of catechins from the milk samples. Caffeine–catechin interactions are well known to produce insoluble complexes known as tea cream visible as haze and a precipitate in brewed tea infusions (Liang, Lu, & Zhang, 2002; Liang & Xu, 2001, 2003). Oily surface films observed in brewed tea are believed to be a result of catechin interaction with calcium (Horie, Ujihara, & Kohata, 2002). It is plausible that catechin complexes such as these could precipitate with the protein fraction and become unrecoverable by standard extraction techniques.

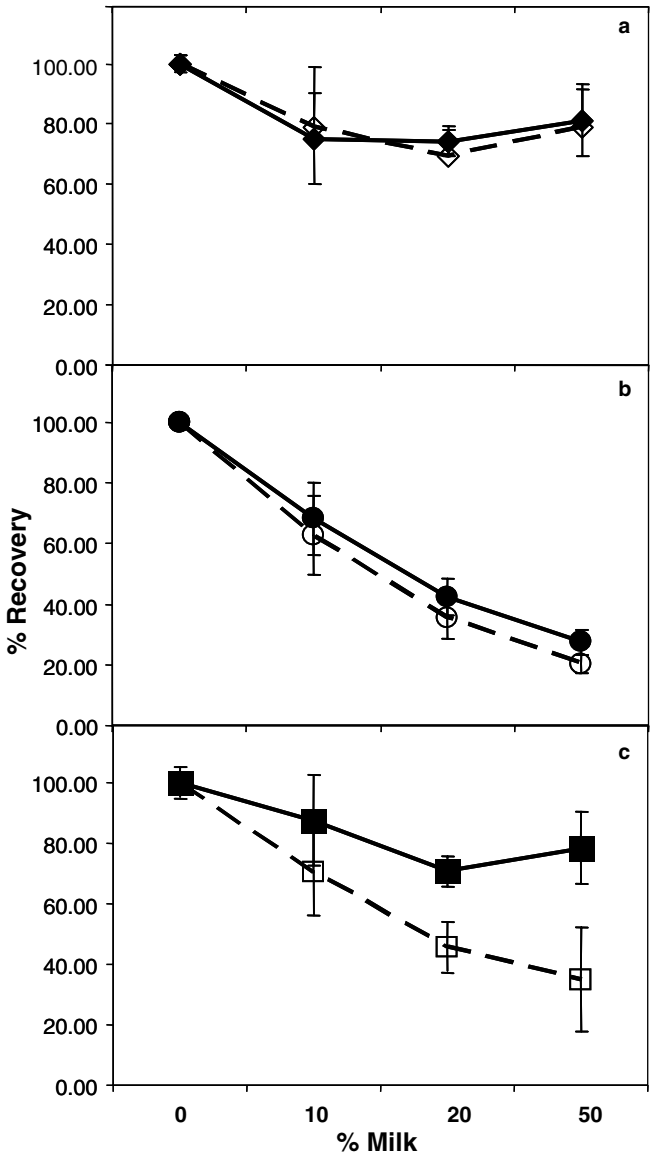

Fig. 3. Effect of milk content on recovery of (a) EC (◇) and EGC (◆) and (b) EGCG (●) and ECG (○) (c) Total catechins (□) and caffeine (■). Data represent percentage of catechins recovered following acid precipitation of corresponding milk tea solutions. Values represent means ± SEM for five independent extractions.

3.3. Effect of extraction method on catechin recovery

In order to improve catechin recoveries observed from acidified aqueous extraction we investigated two alternative methods with the intent to destabilize potential catechin–protein association: methanol treatment and pepsin hydrolysis. Ethanol and/or methanol treatment is typically used in dissociation of many bioactive compounds, such as retinol, with binding proteins (Bychkova, Dujsekina, Fantuzzi, Ptitsyn, & Rossi, 1998; Craft et al., 2000). Considering the high solubility of catechins in methanol and its common use as an extraction solvent from tea infusions and leaf (Yao et al., 2004) it represented a logical extraction solvent for tea catechins from milk matrices. Pepsin was

selected as protease enzyme for enzyme assisted extraction based on its high specific activity at low pH (<3.0). Low reaction pH is favourable to ensure maximal catechin stability as catechins are well known to be unstable at pH 7 and above (Zhu, Zhang, Tsang, Huang, & Chen, 1997).

For methanol extractions, 2 mL aliquots of milk–tea solutions were combined with 6 mL of methanol and mixed vigorously by vortexing. Following centrifugation, the liquid fraction was quantitatively collected and residual precipitate re-extracted with 6 mL of methanol. This method resulted in an increase of total catechin recovery to 85% and 81% for 20% and 50% milk–tea solutions, respectively (Fig. 4). These recoveries represent a very highly significant ($p < 0.001$) improvement over 45% and 26% recovery from simple acidified water extraction of 20% and 50% milk–tea, respectively.

Enzyme assisted extractions with pepsin were accomplished by combining 2 mL aliquots of milk–tea solutions with 6 mL of 0.1 N HCl containing 40 mg/mL pepsin. Solutions were mixed gently and incubated for 15 min at 37 °C in a shaking water bath. A preliminary investigation determined that a 15 min enzyme treatment was adequate for effective enhancement of catechin recovery for our samples. Pepsin treatment longer than 15 min did not result in further improvement of analytical recoveries (data not shown). Following incubation, aliquots were collected for analysis. Total catechin recovery was enhanced to 96.5% and 95.6% for 20% and 50% milk–tea solutions, respectively (Fig. 4). This recovery was found to be significantly higher than that observed from both methanol ($p < 0.05$) and acidified water ($p < 0.001$) techniques. Improvement observed from the use of methanol compared to acid precipitation are likely due to the different polarities and solvent strength of methanol compared to acidified water. However, some degree of interaction between catechin and intact protein is possible and may result in incomplete extraction by methanol. Further improvements to catechin recovery observed by pepsin treatment demonstrate that hydrolysis is advantageous in its ability to further release protein bound catechins. Enzymatic hydrolysis prior to methanol extraction was found to raise overall catechin recovery levels to that of pepsin hydrolysis followed by aqueous extraction (data not shown).

Overall recovery improvement was greatest for gallated catechin forms EGCG and ECG (Fig. 5). Gallated catechins such as EGCG and ECG have previously been shown to have the strongest association with milk protein. These associations may further impact biological effects such as antioxidant and antimutagenic activities often attributed to tea catechins (Arts et al., 2002; Catterall et al., 2003). Effective recovery of EGCG is essential to proper analytical assessment of tea catechins as it is the most abundant catechin in tea representing almost 50% of total catechin content in the green tea infusions utilized in this study. In 50% milk–tea solutions, EGCG recovery was improved from 27% with acidified water extraction to 94% by pepsin

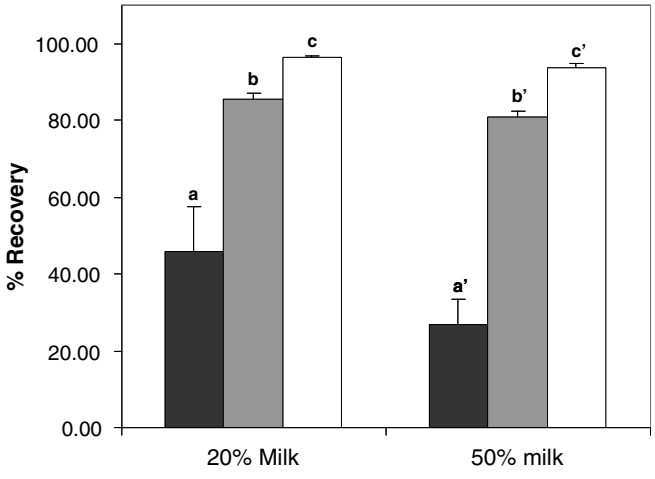

Fig. 4. Recovery of total catechins from 20% and 50% milk–green tea solutions. Data represent percentage of catechins recovered following either acid precipitation (■), methanol extraction (▩), or enzyme assisted extraction (□) from corresponding milk tea solutions. Values represent means ± SEM for five independent extractions. Different letters represent significant differences in catechin recovery by methanol extraction ($p < 0.05$), and enzyme assisted extraction ($p < 0.001$).

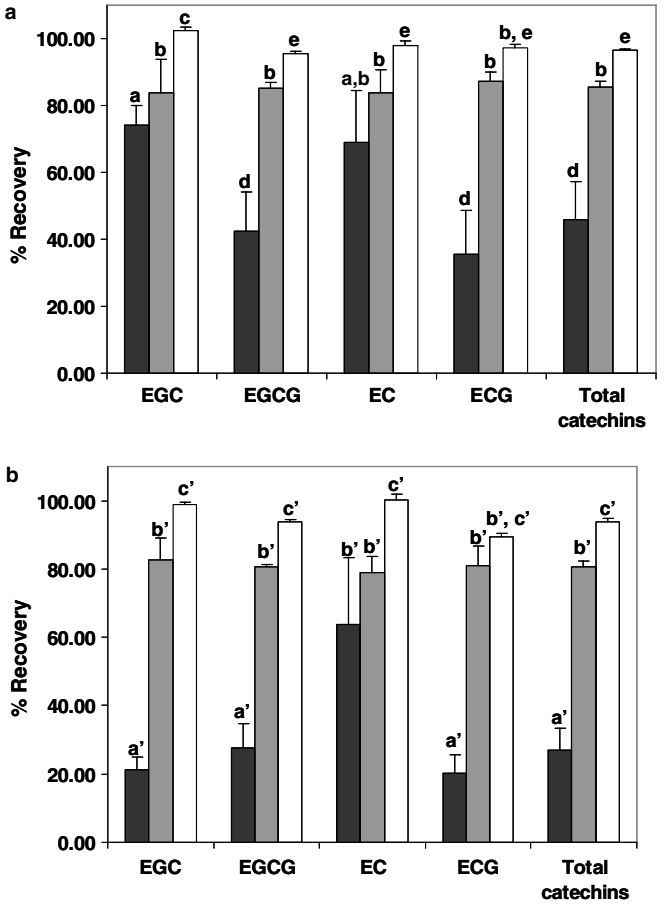

Fig. 5. Recovery of total catechins and caffeine from (a) 20% milk–green tea and (b) 50% milk–green tea solutions. Data represent percentage of catechins recovered following either acid precipitation (■), methanol extraction (▩), enzyme assisted extraction (□) from corresponding milk tea solutions. Values represent means ± SEM for five independent extractions. Different letters represent significant differences in individual and total catechin extraction recoveries.

treatment. Methanol extraction of 50% milk–tea solutions resulted in only an 80% recovery of EGCG clearly demonstrating the benefit of pepsin treatment in extraction of EGCG from these samples.

While methanol extraction is simple and commonly utilized for flavonoid analysis, there are several advantages to application of enzyme assisted extraction as outlined in this study. First, conditions outlined in our enzyme assisted extraction of catechins accounts for many aspects of catechin instability and artifact formation. Specifically, extraction time, sample pH and direct compatibility with highly aqueous LC analysis. The short 15 min reaction time utilized for pepsin hydrolysis is practical and minimizes chance of side reactions which may occur during long incubation times. Catechin stability is known to be optimal at low pH (Zhu et al., 1997) which corresponds to conditions of pepsin hydrolysis employed in this study (pH 2.5–3.0). Exposure to elevated pH (>7.0) for even short periods of time result in significant and potentially total loss of monomer catechins through polymerization reactions (Haslam, 2003; Yoshino, Suzuki, Sasaki, Miyase, & Sano, 1999). Finally, enzyme treated samples are readily compatible for dilution into mobile phase, filtration and direct injection onto HPLC minimizing unnecessary solvent extraction, concentration and drying steps that potentially introduce variability. Compatibility with a high aqueous mobile phase is critical in order to optimize LC separation conditions and avoid peak artifacts. Methanol and other solvent extracts require dilution or solvent evaporation prior to analysis in order to achieve optimal chromatographic separation in turn increasing sample handling and opportunity for further degradation and artifact formation.

3.4. Analytical precision

Intra-day variability expressed as coefficient of variation (CV) was determined for each catechin extracted by enzyme assisted extraction. Two millilitre aliquots of 50% milk samples were extracted in triplicate and analyzed by HPLC as described previously. Intra-day variability for EC, EGC, EGCG, ECG were found to be 0.83%, 0.76%, 1.60% and 1.08%, respectively, representing excellent precision for this method.

3.5. Analysis of commercial products

Analysis of commercially available tea–milk beverages was undertaken to demonstrate the versatility of enzyme assisted extractions for analysis of various liquid matrices which have been subjected to UHT and/or retort processing. Catechin contents for both RTD black tea chai latte and green tea–soymilk beverages can be seen in Fig. 6. As with model green tea–milk system all three extraction techniques (acid precipitation, methanol extraction, and enzyme assisted extraction) were performed for comparison purposes. Fig. 6(a) shows the catechin content of the RTD green tea–soymilk beverage. Content of EC, EGCG and EGC were similar for all methods illustrating similar extraction efficiencies. Total catechin content ranged from 35 mg/250 mL with methanol extraction to 60 mg/250 mL as assayed with enzyme assisted extraction. Acid precipitation results were similar to methanol extraction. Observed differences were likely due to enhanced extraction of ECG by enzyme assisted methods compared to acid or methanol extractions resulting in a significant ($p < 0.01$) 73% increase in total measured catechin content.

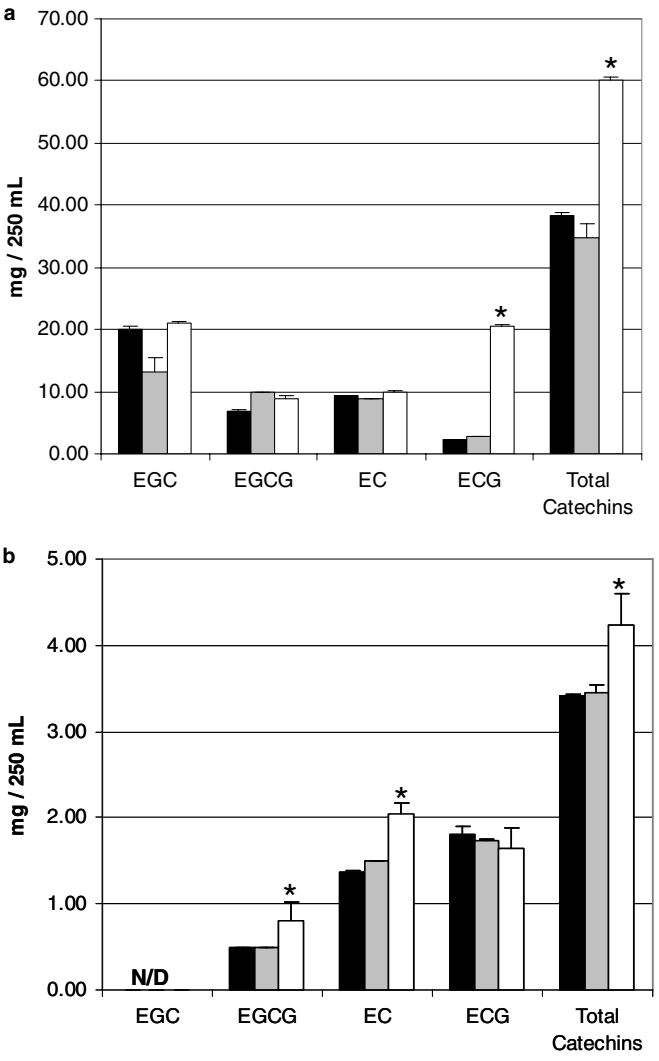

Fig. 6. Catechin contents for (a) commercial green tea–soymilk RTD beverage and (b) RTD black tea chai latte extracted by either acid precipitation (■), methanol extraction (■), enzyme assisted extraction (□). Values represent means ± SEM for three independent extractions. The presence of an asterisks (*) above the error bars indicates that recovery of ECG and total catechins from green tea–soymilk beverage as well as EGCG, EC and total catechins from RTD black tea chai latte were significantly higher for enzyme assisted extractions than from methanol ($p < 0.05$) or acid precipitation methods ($p < 0.01$).

Catechin content of a RTD black tea chai latte is shown in Fig. 6(b). As with the green tea–soymilk beverage, total catechin content assayed by enzyme assisted extraction was determined to be higher than with methanol extraction or acid precipitation methods. As is common for black tea

samples total catechin levels were lower than for green tea ranging from 3.4 to 4.3 mg/250 mL. Overall low levels are likely due to selection of black tea, low overall tea content and thermal degradation of tea catechins during thermal processing of this product. Specific differences were observed in EC and EGCG content with the enzyme assisted method providing higher values. EGC was not detected in this black tea product. This is most likely due to low levels of EGC in black tea and further loss due to thermal process of this RTD product. While providing a statistically significant ($p < 0.05$) 27% increase in total assayed catechin content, the absolute difference is small considering the low total catechin content in this product.

4. Conclusions

Analysis of tea catechins is critical to development and clinical assessment of new tea products. Combination of tea with milk and subsequent catechin–protein interactions create a difficult matrix for traditional extraction techniques. Previously, application of enzyme hydrolysis has been used primarily for release of catechins from their conjugated forms (Chu et al., 2004). In the present investigation, a short-time pepsin treatment was utilized to sufficiently digest the protein fraction to disrupt the catechin–protein interactions and enhance their recovery from milk matrices. This enzyme assisted extraction method proved to be a more suitable method for recovery and direct analysis of catechins from various milk tea beverages. It is our intent to expand upon this work by further exploring the effect of thermal treatment on tea polyphenol recoveries from dairy based products and further refining methodology to provide for accurate and reliable measurement of physiologically significant tea polyphenols in dairy and soy based beverage systems.

References

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

Arts, I. C. W., & Hollman, P. C. H. (1998). Optimization of a quantitative method for the determination of catechins in fruits and legumes. *Journal of Agriculture and Food Chemistry, 46*, 5156–5162.

Baxter, N. J., Lilley, T. H., Haslam, E., & Williamson, M. P. (1997). Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry, 36*, 5566–5577.

Borrelli, F., Capasso, R., Russo, A., & Ernst, E. (2004). Systematic review: green tea and gastrointestinal cancer risk. *Alimentary Pharmacology and Therapeutics, 19*, 497–510.

Bychkova, V. E., Dujsekina, A. E., Fantuzzi, A., Ptitsyn, O. B., & Rossi, G. L. (1998). Release of retinol and denaturation of its plasma carrier, retinol-binding protein. *Fold and Design, 3*(4), 285–291.

Catterall, F., Kassimi, A. I., Clifford, M. N., & Ioannides, C. (2003). Influence of milk on antimutagenic potential of green and black teas. *Anticancer Research, 23*, 3863–3868.

Chandra, S., & Gonzalez de Mejia, E. (2004). Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of *Ardisia compressa* in comparison to mate (*Ilex paraguariensis*) and green (*Camellia sinensis*) teas. *Journal of Agriculture and Food Chemistry, 52*, 3583–3589.

Chu, K. O., Wang, C. C., Chu, C. Y., Rogers, M. S., Choy, K. W., & Pang, C. P. (2004). Determination of catechins and catechin gallates in tissues by liquid chromatography with coulometric array detection and selective solid phase extraction. *Journal of Chromatography B, 810*, 187–195.

Craft, N. E., Haitema, T., Brindle, L. K., Yamini, S., Humphrey, J. H., & West, K. P., Jr. (2000). Retinol analysis in dried blood spots by HPLC. *Journal of Nutrition, 130*, 882–885.

Geleijnse, J. M., Launer, L. J., van der Kuip, D. A. M., Hofman, A., & Witteman, J. C. M. (2002). Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. *American Journal of Clinical Nutrition, 75*, 880–886.

Haslam, E. (2003). Review: thoughts on thearubigins. *Phytochemistry, 64*, 61–73.

Henning, S. M., Fajardo-Lira, C., Lee, H. W., Youssefian, A. A., Go, V. L. W., & Heber, D. (2003). Catechin content of 18 teas and a green tea extract supplement correlates with the antioxidant capacity. *Nutrition and Cancer, 45*(2), 226–235.

Higdon, J. V., & Frei, B. (2003). Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Critical Reviews in Food Science and Nutrition, 43*, 89–143.

Hollman, P. C., Feskens, E. J., & Katan, M. B. (1999). Tea flavonols in cardiovascular disease and cancer epidemiology. *Proceedings of the Society of Experimental Biology and Medicine, 220*, 198–202.

Horie, H., Ujihara, T., & Kohata, K. (2002). Oily film formed on the surface of the oolong tea infusion. *Journal of the Japanese Society for Food Science and Technology (Nippon-Shokuhin-Kagaku-Kogaku-Kaishi), 49*(5), 345–347.

Jöbstl, E., O'Connell, J., Fairclough, J. P. A., & Williamson, M. P. (2004). Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules, 5*, 942–949.

Kammerer, D., Claus, A., Carle, R., & Schieber, A. (2004). Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera*) L. by HPLC-DAD-MS/MS. *Journal of Agriculture and Food Chemistry, 52*, 4360–4367.

Lee, B.-L., & Ong, C.-N. (2000). Comparative analysis of tea catechins and theaflavins by high performance liquid chromatography and capillary electrophoresis. *Journal of Chromatography A, 881*, 439–447.

Lee, M.-J., Prabhu, S., Meng, X., Li, C., & Yang, C. S. (2001). An 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.

Liang, Y., Lu, J., & Zhang, L. (2002). Comparative study of cream in infusions of black tea and green tea [*Camellia sinensis* (L.) O. Kuntze]. *International Journal of Food Science and Technology, 37*(6), 627–634.

Liang, Y., & Xu, Y. (2001). Effect of pH on cream particle formation and solids extraction yield of black tea. *Food Chemistry, 74*, 155–160.

Liang, Y., & Xu, Y. (2003). Effect of extraction temperature on cream and extractability of black tea [*Camellia sinensis* (L.) O. Kuntze]. *International Journal of Food Science and Technology, 38*(1), 37–45.

Manning, J., & Roberts, J. C. (2003). Analysis of catechin content of commercial green tea products. *Journal of Herbal Pharmacotherapy, 3*(3), 19–32.

Mukamal, K. J., Maclure, M., Muller, J. E., Sherwood, J. B., & Mittleman, M. A. (2002). Tea consumption and mortality after acute myocardial infarction. *Circulation, 105*, 2476–2481.

Murray, N. J., Williamson, M. P., Lilley, T. H., & Haslam, E. (1994). Study of the interaction between salivary proline-rich proteins and a polyphenol by ^{1}H-NMR spectroscopy. *European Journal of Biochemistry, 219*(3), 923–935.

Papadopoulou, A., Green, R. J., & Frazier, R. A. (2005). Interaction of flavonoids with bovine serum albumin: a fluorescence quenching study. *Journal of Agriculture and Food Chemistry, 53*, 158–163.

Sakakibara, H., Honda, Y., Nakagawa, S., Ashida, H., & Kanazawa, K. (2003). Simultaneous determination of all polyphenols in vegetables, fruits, and teas. *Journal of Agriculture and Food Chemistry, 51*, 571–581.

Scalbert, A., Manach, C., Morand, C., Remesy, C., & Jimenez, L. (2005). Dietary polyphenols and the prevention of disease. *Critical Reviews in Food Science and Nutrition, 45*, 287–306.

Sesso, H. D., Gaziano, J. M., Buring, J. E., & Hennekens, C. H. (1999). Coffee and tea intake and the risk of myocardial infarction. *American Journal of Epidemiology, 149*, 162–167.

Siebert, K. J., Troukhanover, N. V., & Lynn, P. Y. (1996). Nature of polyphenol–protein interactions. *Journal of Agriculture and Food Chemistry, 44*, 80–85.

Yang, Y. C., Lu, F. H., Wu, J. S., Wu, C. H., & Chang, C. J. (2004). The protective effect of habitual tea consumption on hypertension. *Archive of Internal Medicine, 164*(14), 1534–1540.

Yao, L., Jiang, Y., Datta, N., Singanusong, R., Liu, X., Duan, J., et al. (2004). HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia. *Food Chemistry, 84*, 253–263.

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

Zhu, Q. Y., Zhang, A., Tsang, D., Huang, Y., & Chen, Z.-Y. (1997). Stability of green tea catechins. *Journal of Agriculture and Food Chemistry, 45*, 4624–4628.