Effect of process unit operations and long-term storage on catechin contents in EGCG-enriched tea drink

Laurent Bazinet a,b,*, Monica Araya-Farias a,b, Alain Doyen a,b, Dominique Trudel c, Bernard Têtuc

a Institute of Nutraceuticals and Functional Foods (INAF), Université Laval, Sainte-Foy (QC), Canada G1V 0A6
b Department of Food Sciences and Nutrition, Pavillon Paul Comtois, Université Laval, Sainte-Foy (QC), Canada G1V 0A6
c Centre de Recherche en Cancérologie, Université Laval, Sainte-Foy (QC), Canada G1V 0A6

A R T I C L E   I N F O
Article history:
Received 19 January 2010
Accepted 15 May 2010

Keywords:
Green tea
Catechins
EGCG
Tea drink
Long-term stability
Process unit operation
Anti-cancer properties

A B S T R A C T

Due to the increasing market for functional foods and the chemopreventive action of (−)-epigallocatechin gallate (EGCG), manufacturers produce ready-to-drink green tea infusions enriched or not in EGCG. However, the maintenance of green tea catechins stability in drinks is always a challenge. In this context, the objectives of this study were (1) to assess the catechin stability in tea drink during a 6-month storage, (2) to evaluate the impact of process unit operations on catechin stability and (3) to compare the catechin and caffeine contents of commercially available tea drinks. It appeared that the stability of catechins during long-term storage was optimum at low temperature (4 °C) and acidic pH (pH 4.0). During the processing of the EGCG-enriched green tea drink, all the process unit operations, except heat-treatment, had no impact on catechin concentrations. In addition, in commercially available tea drinks, except enriched green tea drinks, their catechin contents are very low to provide health benefits.

1. Introduction

EGCG is regarded as the most important of the tea catechins (Ju, Lu, Lambert, & Yang, 2007; Yang & Landau, 2000), due to its high content in tea and recent demonstration of its protective action against cancer (Adhami, Ahmad, & Mukhtar, 2003; Lambert & Yang, 2003; Yu, Yin, & Shen, 2004; Zaveri, 2006). Hence, manufacturers try to produce and package ready-to-drink green tea infusions in different packaging such as cans or bottles, enriched or not in (−)-epigallocatechin gallate (EGCG) or other polyphenols, to respond to the increasing market for functional foods having potential health benefits (Zhao, Yang, & Wang, 2009). However, the production of green tea beverages was found to be problematic (Kim et al., 2007; Wang, Helliwell, & You, 2000). Indeed, EGCG has to be stable in green tea brews and commercial tea drinks to preserve its chemopreventive activity. According to the literature, the stability of EGCG and more generally green tea catechins in solutions and drinks is always a challenge and very few studies on the subject are reported. Su, Leung, Huang, and Chen (2003) observed that green tea catechins were partially decomposed by elevation of temperature and pH of incubation media. Kim et al. (2007) reported that the concentration of total catechins in green tea decreased after a thermal treatment of sterilization. It was also found that tea catechins were sensitive to heat as they were vulnerable to degradation and isomerisation during heat processing and storage (Kim et al., 2007, Ito et al., 2003).

In commercial tea based soft drinks, Chen, Xhu, Tsang, and Huang (2001) observed that catechins showed varying stability with (−)-epigallocatechin gallate (EGCG) and (−)-epigallocatechin (EGC) being more unstable than (−)-epicatechin (EC) and (−)-epicatechin gallate (ECG). The qualitative and quantitative composition of catechin isomers in beverages can vary according to the heat-sterilization conditions as epimerization of tea catechins occurs under heating conditions (Seto, Nakamura, Nanjo, & Hara, 1997). As reported by some studies, approximately 50% of the tea catechins in the marketed green tea beverages are epimerized by heat treatment (Chen et al., 2001; Kim et al., 2007). In addition, the rate of degradation of the green tea catechin is reported to be very variable according to the composition in catechin content (Chen et al., 2001; Sang, Lee, Hou, & Yang, 2005) or to the presence of other compounds such as citric acid or metal ions (Chen et al., 2001; Sang et al., 2005; Wang, Zhou, & Wen, 2006). Consequently, catechins were not expected to be stable over more than few days (Wang & Helliwell, 2000) or weeks (Ito et al., 2003; Su
et al., 2003) according to the conditions of production and temperature of storage.

Since there is limited information on long-term stability of green tea catechins in canned and bottled tea drinks (Su et al., 2003), the main objectives of this study were to produce an EGCG-enriched tea drink at a semi-pilot scale, comprising the different process unit operations (brewing, centrifugation, filtration, pasteurization and bottling) found in the industry to produce such a beverage and then to (1) study the stability of its catechins during a 6-month storage, (2) evaluate the impact of the different process unit operations on the stability of catechins during its production, (3) compare its catechin and caffeine contents with commercially available tea drinks, and finally (4) to evaluate its in-vitro anti-cancer activity.

2. Materials and methods

2.1. Materials

2.1.1. Green tea

The green tea used in this experiment was a commercially available and non-organic Japanese green tea obtained from local retailer. The green tea is composed of particles obtained from coarsely crushed folded-leaf, ranging from 0.4 to 1.5 cm-length. The green tea was harvested in 2007. Before being used, the green tea was stored in vacuum bags at room temperature in a dark and dry space.

2.1.2. Catechin and caffeine standards

The standards for (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, (-)-gallocatechin gallate and caffeine were bought from Sigma-Aldrich (St-Louis, MO, USA).

2.1.3. Commercial tea drinks

Commercial green and black tea beverages, in polyethylene terephthalate (PET or PETE) bottles or aluminium cans were purchased from several local markets in Quebec City (QC, Canada).

2.2. Methods

2.2.1. Production process of the EGCG-enriched tea drink

The EGCG-enriched tea drink was produced at a volume of 50 L, in conditions and with unit operations similar to the ones met in the food industry for the production of low acid beverages (Fig. 1). The first extraction was done in a 150 L stainless steel double-jacket heated reservoir and was performed at 30 °C in 50 L tap water. At the end of the 1st brewing step, the water was discarded (but samples were kept for analysis) while the green tea leaves were gently squeezed to extract remaining water. For the second brewing step, the leaves were added to another 50 L of pre-heated tap water previously heated at 80 °C in the same double-jacket heated reservoir. The brewing was maintained at 80 °C to produce the EGCG-enriched tea drink. The green tea leaves were squeezed and rejected. The 50 L production of EGCG-enriched tea drink

![Diagram of the manufacturing process of the EGCG-enriched tea drink](image-url)

Fig. 1. Scheme of the manufacturing process of the EGCG-enriched tea drink.
was divided in two; one half was pasteurized and bottled directly at the natural pH of the green tea brewing, close to pH 6.0, while the pH of the second half was adjusted to a pH value close to 3.8–4.0 by the addition of commercial concentrated lemon juice. The value of 3.8–4.0 was chosen to eliminate the possibility of *Clostridium botulinum* growth during storage, which is a very potent and dangerous microorganism for human health (Gibson & Roberts, 1986; Lund, Graham, & Franklin, 1987; Vera et al., 2007). As for the non adjusted EGCG-enriched tea drink, the adjusted pH EGCG-enriched tea drink was then pasteurized at 90°C for 30 s and cooled at a final temperature of 4–8°C in a actijoule heat-exchanger (Actinii S.A., Maxilly, France) connected to an aseptic bottling system composed of a combined sterile product outlet and an automatic filling control (Microthermics, Raleigh, NC, USA) built-in a table top laminar flow workstation (model EL 422 TS, Atmos-Tech Industries, Ocean, NJ, USA). Both EGCG-enriched teas were in 500 mL HDPE plastic bottles (Elnova, Rought, QC, Canada) and stored in different conditions.

### 2.2.2. Protocols

#### 2.2.2.1. Catechin stability during long-term storage.

Three 50 L batches of EGCG-enriched tea drink were produced at three different times for the needs of the experiment. One kilogram of green tea leaves was in 50 L of double-distilled water; the 1:50 tea/water ratio (or 20 g of leaves/L of water) and the durations of each brewing step (20 min. at 30°C and 30 min. at 80°C) were used according to our previous experiments (Bazinet, Labbé, & Tremblay, 2007; Labbé, Tremblay, & Bazinet, 2006; Labbé, Tétu, Trudel, & Bazinet, 2008). Bottles of adjusted and non adjusted EGCG-enriched teas were kept in the dark and stored at 4°C and 25°C. HPLC and microbiological analyses were carried out after 0, 15, 30, 60, 90, 120, 150 and 180 days of storage in the different conditions. A 180 days or 6 months stability or nutritional and composition stability during storage is generally aimed by beverage manufacturers.

#### 2.2.2.2. Effect of process unit operation on catechin stability.

Since a clinical study was planned and now currently under way to test the EGCG-enriched tea drink on the maintenance of complete remission in woman with advanced ovarian cancer, and since a 600 mg/L EGCC was aimed to meet the data reported in the literature as effective against cancer (Bettuzzi et al., 2006), the ratio tea/water was adjusted from 20 to 35 g/L. In the same time, the durations of the first brewing step was decreased from 20 to 10 min. and increased for the second step from 30 to 80 min. to increase the diffusion of EGCC. Samples of 10 mL were taken at different times along the process of 50 L production to follow the evolution of catechin after each unit operation (1st and 2nd brewings, centrifugation, filtration, pasteurization and bottling). Fifty-liter brewing condition was repeated twice and samples were all cooled quickly and immediately analyzed by HPLC.

#### 2.2.2.3. Comparison of catechin and caffeine contents with tea beverages commercially available in Quebec.

The experimental EGCG-enriched tea drink used in this experiment and for the clinical trial was compared to tea beverages enriched or not in polyphenols commercially available in the city of Quebec and representative of the Canadian tea beverage market. The different tea beverages were compared in terms of catechins and caffeine concentrations. In this protocol, the durations of the second brewing step was adjusted from 80 to 60 min. in order to obtain a final concentration of EGCC close to the 600 mg/L needed for the clinical trial.

### 2.2.2. HPLC method

Each green tea sample collected during brewing under different conditions was filtered through a 0.20 μm filter (Aerodisc LC13 PVDF, Gelman Laboratory, Ann Arbor, MI) and diluted by a factor of ten with HPLC grade water to be analyzed. The mobile phases were filtered through a 0.20 μm nylon filter (Mandel Scientific Company, Guelph, ON, Canada). The column temperature was maintained at 25°C during analyses and autosampler temperature was kept at 4°C. The detection of analytes was performed with UV detection at 210 nm. Standard curves were calculated from a mix of catechin and caffeine standards at different concentrations: Correlations obtained ranged from 0.99663 to 0.99979. The pump used was a WatersSt 600 pump, the detector was a WatersSt 486 Tunable Absorbance Detector, the autosampler was a Waters 717 plus one and the software was Millennium32 v3.20 (Waters Inc., Lachine, QC, Canada). The column used was an YMC-Pack ODS-AM column, 5-5 μm, 12 nm (YMC Inc., Milford, MA, USA) and solvents were water + 0.05% trifluoroacetic acid (TFA, purity >99%, Laboratoire MAT, Québec, QC, Canada) for phase A and ace-tonitrile (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ, USA) + 0.05% TFA for phase B. All other parameters of the HPLC method were the same as those used by Labbé, Araya-Farias, Tremblay, and Bazinet (2005) which were based on the National Institute of Standards and Technology method (Dalluge, Nelson, Thomas, & Sander, 1998).

### 2.2.4. Microbiological analysis

The number of viable bacteria were determined by a standard plate count in Brain Heart Infusion (BHI) Petri dish with the series dilution method. One-milliliter of EGCG-enriched green tea drink (dilution 10^6) was mixed with 9 mL of sterile distilled water to obtained dilution 10^-1 and further diluted to obtain 10^-2 and 10^-3 dilutions. For all these dilutions, 1 mL was put in different Petri dishes and a BHI broth, containing 15 g/L of agar, was poured and homogenized. The Petri dishes were incubated at 30°C during 72 h with microbiological counts every 24 h. These microbiological counts at 30°C allow the growth of total facultative aero-anaerobic mesophile bacteria (Siqueira & de Uzeda, 1996; van Spreeks & Stekelenburg, 1986; Yuste, Mor-Mur, Capellas, & Pla, 1999).

According to the microbiological standards in Quebec concerning the elaboration of pasteurized beverages elaborated in factory, the green tea drink was considered as safe when the microbiological count was lower than 10^3 org/mL, while when the count was over 10^5 org/mL, the product was considered as unsafe and improper for consumption (Ministère de l’agriculture, 2006).

### 2.2.5. In vitro anti-cancer activity

Anti-cancer assays were realized on the EGCG-enriched tea drink previously prepared from 35 g/L tea/water ratio and adjusted to pH 3.8–4.0 as used for the clinical trial. The tea drink was tested at different concentrations (1:1, 1:10, 1:20, 1:40, 1:80 and 1:160 dilution factors), on four immortalized cancerous cell lines, A549 (lung), HCT15 (colon), BT549 (breast) and PC3 (prostate), representative of the mostly frequent cancers in human (Weisburger, 1985). The tests were performed in microplates of 96 wells and the conditions were realized in duplicate wells in two independent assays. As positive control, the apoptotic agent etoposide was tested at different concentrations (1:1, 1:5, 1:25, 1:125, 1:625 and 1:3125). As negative control, the water which represents the solvent dissolving tests compound was used. Moreover, culture medium was used also as negative control and represented the basal condition of cell growth. The exposition to test compounds with cells was done during 72 h. After this exposition time, detection was performed by luminescence reader for quantification of the anionic dye sulforhodamine B. Sulforhodamine B was used to determine the total protein content measurements of various treated conditions. The
amount of luminescence was directly proportional to the number of living cells in cultures (Monks et al., 1991; Rubinstein et al., 1990; Skehan et al., 1990).

2.2.6. Statistical analyses

Data obtained on catechin and caffeine stability during a 6 month-storage were subjected to one-way analyses of variance using SigmaPlot software (Version 11, Systat, Chicago, IL, USA). Tukey tests were also performed on data using SigmaPlot software. The tukey test was used for all pairwise comparisons of the mean responses to the different treatment groups \( P = 0.05 \).

The data of catechin and caffeine concentrations during process unit operation and for commercially available tea drink, as well as cell viability obtained for in vitro anti-cancer activity were subjected to a one way analysis of variance using SigmaPlot software. The statistical differences between groups or means were determined also using the Tukey test.

3. Results and discussion

3.1. Catechin stability during long-term storage

The one-way analyses of variances, of the catechin concentrations between the beginning and the end of the storage, showed that there were significant differences in ECG (\( P = 0.040 \)), EGC (\( P = 0.012 \)), GCG (\( P = 0.021 \)) and ECG (\( P < 0.001 \)) concentrations during the storage of EGCG-enriched green tea drinks as a function of pH and temperature conditions. The concentrations of caffeine (\( P = 0.0651 \)) and EC (\( P = 0.526 \)) were not affected by the different storage conditions.

During the storage of the EGCG-enriched green tea, the EC and caffeine concentrations were quite stable whatever the pH and temperature conditions at respective values of 43.9 ± 12.5 and 143.4 ± 28.7 μg/mL (Figs. 2 and 3). However, according to the pH and temperature conditions the storage durations were different due to the microbial contamination (Table 1). Indeed, after 60 days of storage at 25 °C, although the levels of catechin were quite stable during this period and that whatever the pH, the measurements of EC and caffeine were stopped due to a high level of contaminants in the tea drinks (Table 1).

For the other catechins, the concentrations varied according to the storage conditions and the type of catechins. For the ECG (Fig. 4) and GCG (Fig. 5), their concentrations were quite stable at 4 °C for both pH values with respective averaged values of 54.3 ± 8.4 and 54.0 ± 7.3 μg/mL during the 180 days storage. However, at 25 °C, their concentrations decreased rapidly to reach values of 0 or close to 0 after 30 days at pH 6 and 30 to 90 days at pH 4. Such a rapid decrease was not observed for EGG (Fig. 6) and EGC (Fig. 7). In fact, at 25 °C, according to the pH, their concentrations decreased between 17% and 43% for ECG after 60 days of storage and between 60% and 68% after 90 days for EGC. At 4 °C, the green tea drink kept a concentration in microorganisms under 30 UFC/mL and was then safe for consumption all along the storage. In the temperature condition of 4 °C, the pH had an impact.

**Table 1** Microbiological count evolution at different incubation durations (24, 48 and 72 h) in EGCG-enriched green tea drink during 180 days-storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25 °C).

<table>
<thead>
<tr>
<th>Time (in day)</th>
<th>4 °C (μg/mL)</th>
<th>25 °C (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.0</td>
<td>pH 6.0</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>15</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>30</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>60</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>90</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>120</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>150</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>180</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>15</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>30</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>60</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>90</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>120</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>150</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>180</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>72 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>15</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>30</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>60</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>90</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>120</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>150</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>180</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
</tbody>
</table>

N/D: not determined.
storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25°C); tea/water ratio of 20 g/L, 1st brewing step at 30°C – 20 min. and 2nd brewing step at 80°C – 30 min.

Fig. 4. Evolution of EGC concentration in EGCG-enriched green tea drink during storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25°C); tea/water ratio of 20 g/L, 1st brewing step at 30°C – 20 min. and 2nd brewing step at 80°C – 30 min.

Evolution of GCG concentration in EGCG-enriched green tea drink during storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25°C); tea/water ratio of 20 g/L, 1st brewing step at 30°C – 20 min. and 2nd brewing step at 80°C – 30 min.

Fig. 5. Evolution of GCG concentration in EGCG-enriched green tea drink during storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25°C); tea/water ratio of 20 g/L, 1st brewing step at 30°C – 20 min. and 2nd brewing step at 80°C – 30 min.

Evolution of ECG concentration in EGCG-enriched green tea drink during storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25°C); tea/water ratio of 20 g/L, 1st brewing step at 30°C – 20 min. and 2nd brewing step at 80°C – 30 min.

Fig. 6. Evolution of ECG concentration in EGCG-enriched green tea drink during storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25°C); tea/water ratio of 20 g/L, 1st brewing step at 30°C – 20 min. and 2nd brewing step at 80°C – 30 min.

Evolution of EGCG concentration in EGCG-enriched green tea drink during storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25°C); tea/water ratio of 20 g/L, 1st brewing step at 30°C – 20 min. and 2nd brewing step at 80°C – 30 min.

Fig. 7. Evolution of EGCG concentration in EGCG-enriched green tea drink during storage in different conditions of pH (4.0 and 6.0) and temperature (4 and 25°C); tea/water ratio of 20 g/L, 1st brewing step at 30°C – 20 min. and 2nd brewing step at 80°C – 30 min.

on the final concentration in EGCG and EGC. The EGCG and EGC concentrations were stable at pH 4 with respective values of 256.0 ± 27.0 and 149.4 ± 27.1 μg/mL while at pH 6, their concentrations decreased slowly, with variations between the beginning and the end of the storage of, respectively 40.0% and 21.6%.

Several studies reported that many factors may affect catechins or more precisely EGCG stability including pH, temperature, oxygen level, antioxidant level, metal ions and the concentration of other ingredients in tea (Chen et al., 2001; Sang et al., 2005; Su et al., 2003; Wang et al., 2006). According to Zhu, Zhang, Tsang, Huang, and Chen (1997) the tea catechins were shown to be extremely unstable in alkaline solution (pH > 8) and degrade completely in a few minutes, while their stability was quite constant in acidic solution (pH < 4). In their study, Su et al. (2003) also found that catechins were more stable at acidic pH rather than alkaline. Concerning the temperature during storages, Demeule et al. (2002) showed that a lower storage temperature extended appreciably catechin half-life. Chang, Zuo, Chow, and Ho (2006) also demonstrated that the temperature of storage markedly influence the stability of phenolic compounds from Hawthorn drink. Frauen, Rode, Steinhart, and Rapp (2000) found that, in cosmetic formulation (oil/water emulsions), catechins decreased to 70% of the initial content at room temperature and only a minimum amount remained at 40°C after 6 months of storage. In the same way, Spanos, Wrolstad, and Heatherbell (1990) observed a complete degradation of epicatechin and catechin in apple juice after storage at 25°C for 9 months. Higher oxygen levels and low concentration of antioxidants increased catechin oxidation while the presence of metal ions enables a metal-catalyzed auto-oxidation of EGCG (Sang et al., 2005). Finally, the presence and concentration of other ingredients such as sucrose, citric acid and ascorbic acid enhanced the degradation of catechins in a solubilized purified green tea catechins extract (Su et al., 2003). However, in the opposite of these previous compounds, a higher catechins concentration was found to positively extend the shelf life and stability of EGCG and other catechins (Sang et al., 2005).

As no significant degradation of the catechins and more precisely EGCG occurred during the 6 months of storage at 4°C, the balance of the factors affecting stability must have been positive. As proposed by Labbé et al. (2008), the use of tap water prevented the presence of extrinsic metal ions in the brewing and the proper precaution taken to prevent excessive headspace in the 500 mL bottles limited oxygen only to the dissolved molecules. An acidic
pH of around 4.0, a storage temperature of 4 °C also contributes in the limitation of the catechins degradations. Finally, as the EGCG-enriched green tea drink is not a pure catechin extract, the presence of other catechins probably highly contributes to the stability of the catechins content. These results confirmed those obtained by Labbé et al. (2008) with no significant degradation of the different catechins during a 8 weeks-storage. They demonstrated in 15 mL falcon laboratory tubes stored at 4 °C in a fridge without any process step such as pasteurisation, bottling or filtration that the catechin content in an EGCG-enriched green tea produced by a two-step extraction process was very stable.

3.2. Effect of process unit operation on catechin stability

The one way analysis of variance demonstrated a significant difference between the different unit operation for the five catechins (P < 0.001 for all five catechins) and caffeine (P < 0.001) concentration during the two-step brewing process.

During the two-step EGCG-enriched green tea production, the concentrations of EGC, caffeine and EC decreased from the 1st infusion step to the 2nd infusion step and then remained constant all along the process at averaged respective concentration of 668.5 ± 6.7, 513.1 ± 8.3 and 131.2 ± 4.1 µg/mL (Table 2). The concentrations of GCG and ECG increased from the 1st brewing to the 2nd brewing step and then were quite constant during the rest of the process with respectively a slight decrease (9.7%) and increase (12.8%) (Table 2). EGCG concentration also increased during the 2nd step brewing. However, its concentration was constant at 916.8 ± 22.2 µg/mL during centrifugation and filtration steps and then decreased of 8.25% after pasteurization and bottling to reach a final concentration of 840.9 ± 1.6 µg/mL (Table 2). In the case of EGCG the change in concentration is more significant than for GCG and ECG; EGCG decrease corresponded to a decrease of 75.9 µg/mL in comparison with respective changes of only 8.6 and 15.7 µg/mL.

The decreases in EGC, EC and caffeine concentrations and increases in GCG, ECG and EGCG between the 1st and 2nd brewing steps were in accordance with previous studies of Labbé et al. (2006), Labbé et al. (2008), and Bazinet et al. (2007). EGC, EC and caffeine were demonstrated to be compounds which are not time/temperature dependent and diffused easily whatever the temperature while GCG, ECG and EGCG were demonstrated to be time/temperature dependant (Labbé et al., 2006). Due to their no time/temperature dependencies, EGC, EC and caffeine diffused mainly during the 1st brewing. Their remaining concentrations were lower in the green tea leaves after the 1st brewing step and consequently their final concentrations in the 2nd brewing were lower. The opposite applied for the GCG, ECG and EGCG. None of the process operation seemed to affect the stability of EGC, EC and caffeine. EGCC was affected by the pasteurization and bottling unit operations; EGCG was probably mainly affected by the pasteurization step since it is well known that this compound is vulnerable to heat treatment (Seto et al., 1997; Su et al., 2003). The decrease in EGCG concentration observed in this study is quite consistent, relatively to the time/temperature pasteurization ratio used (30 s/90 °C) with data reported in the literature. Stach and Schmitz (2001) reported degradation of 14% and 21% for EGCG and EGCG after 60 min of brewing at 90 °C. Su et al. (2003) also observed that 29% and 25% of the total green tea catechins were destroyed when heating for 3 h respectively at 70 °C and 100 °C. In addition, Ito et al. (2003) studied green tea brewing with a heat-pretreatment at 80 °C up to 120 min. and observed decreases in EC but did not measure or take in account the EGCG molecule.

No information was found in the literature concerning the influence of the other process unit operations on catechins evolution. The main works reported in the literature were dedicated to the study of catechins stability during storage (Zhao et al., 2009), during brewing or after brewing (Ito et al., 2003; Stach & Schmitz, 2001; Wang, 2000; Zhao et al., 2009) or on tea drinks in can or bottle at the end of the processing (Su et al., 2003; Yao et al., 2006).

In our process, the maximum brewing temperature used was 80 °C, which would preserve the main part of the EGCG since temperature over 90 °C during brewing were reported to affect this compound (Labbé et al., 2008; Perva-Uzunalic et al., 2006; Su et al., 2003). In addition, the pasteurization heat treatment, although carried-out at 90 °C was quite short (30 s) to affect slightly the EGCG. This process is consequently a good process to ensure the inoacity of the product, as demonstrated previously by the long-term study, as well as the stability of our catechin compounds.

3.3. Comparison of catechin and caffeine contents with commercial tea drinks

The one-way analyses of variance demonstrated significant differences between the 10 commercial tea beverages for the five catechins (P < 0.001 for all five catechins) and the caffeine (P < 0.001) concentrations.

The experimental two-step EGCG-enriched green tea beverage has the highest contents in catechin and caffeine while the Tetley iced green tea and the Nestlé Iced black tea had the lowest one with concentrations of 0 µg/mL for all compounds (Table 3). As expected, the Snapple iced black tea and Lipton Pure leaf (black tea) showed concentrations of 0 µg/mL for all five catechins and respective concentrations of caffeine of 0 and 95.4 µg/mL (Table 3). The six remaining green teas had intermediary caffeine contents ranging from 99.5 to 157.4 µg/mL (Table 3). Amongst these six teas, the Urban Zen teas «ginger juice» and «honey and jasmine», as well as Snapple Lime green tea had similar catechin contents except in EC. The Urban Zen tea «Lemon juice», and the polyphenol-enriched Nestea teas had also similar catechin values except for GCG, where polyphenol-enriched teas presented GCG concentrations of 0 µg/mL in comparison with 99.6 µg/mL for the Urban Zen tea. Major differences were observed between tea beverages in terms of ECG and GCG. Only 4 tea drinks (experimental green tea, Urban Zen tea «Lemon juice», and both polyphenols...
enriched Nestea teas) contained ECG and at concentrations ranging between 19.1 and 111.1 µg/mL (Table 3). For GCG, only five teas (experimental, the three Urban Zen tea and the Snapple Lime green teas) demonstrated valuable content ranging from 33.6 to 169.8 µg/mL. In addition, it appeared from these data that the experimental tea has total polyphenol and EGCG (the main interesting catechin compounds) contents at least 4 and 4.5 times higher respectively than the highest ones of green tea drinks commercially available.

It appeared from these results that as expected, black teas did not contain any catechins since in their process of production from green tea, the black tea are fermented so that catechins are oxidised and polymerized (Lakshminarayanan & Ramaswamy, 1978; Muthumani & Kumar, 2007). The fact that the Nestlé iced black tea did not contain any caffeine is probably due to a decaffeination of the tea. What is surprising is that the Tetley iced green tea did not contain any catechins and caffeine. Since the particular conditions of each tea drink processes are not known it is difficult to discuss the reason of these differences. However, based on the works of Labbé et al. (2006), Labbé et al. (2008), and Bazinet et al. (2007), the highest concentrations of GCG observed in the Urban Zen teas, the Snapple Lime green tea and the experimental one could be probably due to highest time/temperature ratios or simply highest temperatures used for brewing the tea in comparison with both Nestea polyphenol-enriched teas. As demonstrated by Chen et al. (2001) the catechin content of green tea or assimilated green tea drinks commercially available was very low in comparison with tea traditionally prepared. These authors concluded that the catechins have been converted to their corresponding epimers during their production/manufacturing. The experimental two-step enriched green tea drink appeared as an interesting source of the EGCG chemopreventive compound in comparison with other teas founds in the market, even if certain green tea beverages were enriched in green tea extracts such as nestea green tea drinks. However, all these tea drinks had high contents of sugars up to 30 g/341 mL.

### 3.4. Anti-cancer activity evaluation

Anti-cancer assays were realized on the EGCG-enriched green tea drink previously prepared from 35 g/L tea/water ratio. It appeared from the results of in vitro anti-cancer activities that the EGCG-enriched tea drink produced in this study is effective on the different cancerous cell lines tested. However, the anti-cancer activity was different according to the cell lines tested and the dilution of the tea drink. In fact, the best anti-cancer activity for the EGCG-enriched tea drink was observed on prostate cancerous cells with a 88.75% inhibition of cancerous cells followed by 80.5% and 80.25% inhibition for lung and colon and 55% inhibition on breast cancerous cells (Fig. 8). These inhibitions were obtained when the tea drink was not diluted. When the tea drink was diluted no significant inhibition rates were obtained whatever the dilution. This could be due to the fact that the active molecules were too diluted to reach a sufficient concentration and to demonstrate one anti-cancer activity; at the first lowest dilution rate (1:10), the concentration in EGCG was decreased from 651 µg/mL (1.4 µM) to 65.1 µg/mL (0.14 µM) and the total catechin content from 1518 to 151.8 µg/mL.

Results obtained in several in vitro studies confirmed our results and have shown that catechins exhibited antiproliferative and growth controlling properties in cancerous and normal cell lines (Nichenametla, Tarusco, Barney, & Exon, 2006). Hence, according to Valcic, Timmermann, and Alberts (1996), EGCG, EGC and GCG have shown to exhibit high antiproliferative action against MCF-7 breast cancer and HT-29 colon cancer cell lines. At 50 µM concentration, GC and GCG have been reported to inhibit the proliferation of human cancer cell lines MCF-7 (breast) by 95 and 97%, HCT-116 (colon) by 85% and 93% and NCI-H460 (lung) by 87% and 67% (Seeram, Zhang, & Nair, 2003). Furthermore, against lung tumor cell lines, H661 and H1299, EGCG and EGC have displayed strong growth inhibitory effects with an IC50 of 22 µM (Williams, Elliott, Perry, & Greaves, 1996). According to these authors, EGCG and EGC were less effective against lung cancer cell line H441 and colon cancer cell line HT-29 with IC50 values greater than 22 µM. EGCG has also been shown to induce apoptosis, cell-growth inhibition and cell cycle deregulation in DU145 human prostate cancer cells (Adhami et al., 2003). More specifically on green tea polyphenols extract (<3% moisture, 43% EGCG, 13% other catechin polyphenols), Steele et al., 2000 obtained on A427 cells (human lung tumor cell line) an inhibition of 74–92%. However, these authors mentioned that green tea extract (caffeinated, freeze-dried hot water extract, 44.06% polyphenols, 11.16% EGCG) showed little inhibition. Nichenametla et al. (2006) concluded in their review of different in vitro studies that the effective concentration of catechins to exert in vitro antiproliferative activity seems to be 20–100 µM and vary in this range according to the type of cell lines.

### Table 3

<table>
<thead>
<tr>
<th>Company name or commercial brand</th>
<th>Product</th>
<th>Compounds concentration (in µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EGC</td>
</tr>
<tr>
<td><strong>University Laval</strong></td>
<td>Experimental(35 g/L, 30 °C – 10 min. and 80 °C – 60 min.)</td>
<td>537.9 ± 42.3abc</td>
</tr>
<tr>
<td><strong>Urban Zen tea</strong></td>
<td>Infused whole leaf green tea – ginger juice</td>
<td>43.4 ± 1.6c</td>
</tr>
<tr>
<td></td>
<td>Infused whole leaf green tea – honey and jasmine – 500 mL</td>
<td>37.4 ± 3.2c</td>
</tr>
<tr>
<td></td>
<td>Infused whole leaf green tea – lemon juice</td>
<td>150.2 ± 10.3b</td>
</tr>
<tr>
<td><strong>Lipton</strong></td>
<td>Pure leaf (black tea), Iced tea – 473 ml</td>
<td>0.0 ± 0.0c</td>
</tr>
<tr>
<td><strong>Snapple</strong></td>
<td>Iced tea (black tea), lemon – 473 mL</td>
<td>0.0 ± 0.0c</td>
</tr>
<tr>
<td><strong>Tetley</strong></td>
<td>Iced tea, Lime green tea – 473 mL</td>
<td>42.9 ± 2.4c</td>
</tr>
<tr>
<td><strong>Nestlé</strong></td>
<td>Iced green tea – 1.89 L</td>
<td>158.8 ± 24.9b</td>
</tr>
<tr>
<td><strong>Nestlé</strong></td>
<td>Iced tea (black tea), lemon – 1 L</td>
<td>150.2 ± 10.3b</td>
</tr>
<tr>
<td><strong>Nestlé</strong></td>
<td>Nestea, with natural lemon flavour – 172 mg polyphenols – 341 mL</td>
<td>129.5 ± 10.2b</td>
</tr>
</tbody>
</table>

A In the same column, figures with different letters are different at a statistical level of 5% or P < 0.05.
and catechins. In addition they mentioned that the in vitro effective concentrations of catechins may not be relevant to those found in human serum. Indeed, in their study on serum levels of catechins in humans consuming eight cups of black tea a day, Steele et al. (2000) showed a rise of catechins in serum from 0.08 to 0.2 μM.

The cancer chemopreventive properties of green tea have been attributed to its inhibition of tumor cell proliferation and molecular pathways involved in the cell cycle, angiogenesis, invasion, and growth factor-related proliferation (Adhami et al., 2003; Lambert & Yang, 2003; Zaveri, 2006). According to many authors, green tea also inhibits angiogenesis and tumor invasion by inhibiting metalloproteinases and the vascular endothelial growth factor receptor expression and signaling in tumor and endothelial cells, respectively (Jung et al., 2001; Kojima-Yuasa, Hua, Kennedy, & Matsui-Yuasa, 2003; Masuda et al., 2002; Waleh, Chao, Bensari, & Zaveri, 2005). Thus, cancer inhibition by catechins seems to be a complex process that needs to be tested in humans. Moreover, despite varying results obtained on cancerous cell lines, epidemiological studies show that green tea consumption delays cancer incidence (Nakachi, Eguchi, & Imai, 2003). Therefore, chemopreventive clinical trials are needed to assess the effects of green tea consumption on human health.

4. Conclusions

It appeared from these results that the stability of catechins during long-term storage was optimum at low temperature
(4°C) and pH (pH 4.0). The pH has also the advantage to preserve the safety of the product against pathogenic microorganisms and germs which could alter the tea quality during storage. In addition, during the processing of the EGCG-enriched green tea drink, the process unit operations had no impact on catechin concentrations except for the heat treatment. In fact, only heat treatment such as pasteurization decreased the catechin concentration and mainly EGCG. Once again, the low pH value minimized the necessity to apply high heat treatment such as sterilization. The EGCG-enriched green tea drink presented high values in catechins in comparison with commercially available tea drinks with thus a potential anti-cancer property. In addition, except for enriched green tea drinks the catechin contents of green tea or assimilated green tea drinks commercially available was very low in comparison with tea traditionally prepared. A clinical study is currently under way to test the EGCG-enriched tea drink, produced in the second step of the two-step extraction procedure, on the maintenance of complete remission in woman with advanced ovarian cancer.

The concentration of EGCG, or total polyphenol is very high in our EGCG-green tea drink in comparison with commercially available green tea drinks, and the safety, in terms of potential pro-cancer properties, of this beverages on human health could be pointed out. However, Chow et al. (2003) concluded that it is safe to take green tea polyphenol products in amounts equivalent to the EGCG content of 8–16 cups of green tea once a day. Furthermore, following high EGCG concentration consumption (800 mg EGCG once a day), they observed a >60% increase in the systemic availability of free EGCG after chronic green tea polyphenol administration. In addition, according to Chow et al. (2003) consumption of up to 20 cups of green tea per day is not uncommon in certain populations. In addition, some studies suggested that the caffeine content of green tea contributes to its biological activity (e.g. UV protection) in comparison with decaffeinated products (Agarwal, Katiyar, & Mukhtar, 1993; Lou, Lu, Xie, Huang, & Conney, 1999; Lu et al., 2001).

Acknowledgements
This work was made possible by the technical assistance of Alain Gaudreau, research professional at Laval University. The natural supports of the Natural Sciences and Engineering Research Council of Canada (NSERC) and Canadian Cancer Society are also acknowledged.

References