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A study of stability of (-)-Epigallocatechin gallate (EGCG) from green tea in a frozen product

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Abstract: Epigallocatechin gallate (EGCG) from green tea has a potential to reduce the risk of cardiovascular disease and cancer. However, EGCG is unstable at high temperature and at pH ≥ 5. Strawberry sorbet is a high acid food product (pH < 4.6) that is stored frozen. The aim of the study is to examine the stability of EGCG in the strawberry sorbet by determining the amount of EGCG immediately after made and stored at 18ºC for up to 16 weeks. In addition, the stability of EGCG in a strawberry sorbet and in distilled water as well as those under simulated intestinal digestion (pH 7.5 and at 37ºC for up to 12 hours) was compared. The data showed that > 90% EGCG was in the strawberry sorbets after storage for 16 weeks. EGCG were also more stable in the strawberry sorbets than in distilled water under the simulated intestinal digestion significantly (p<0.05).

Keywords: Green tea, (-)-Epigallocatechin gallate (EGCG), catechins, strawberry sorbet

Introduction

Green tea has recently become more popular because of the health benefits attributed to its infusion. A number of epidemiological studies in Japan and China have shown that people who regularly drink green tea have less heart disease risks and certain cancers conditions (Sano *et al*., 2004; Le *et al*., 2006; Kuriyama *et al*., 2006). Green tea is rich in catechins. Many studies indicate that the catechins have a number of health promoting properties including anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-bacterial, anti-obesity and anti-atherosclerotic (heart disease and stroke) effects (Koo and Cho, 2004). Relative to heart disease and stroke prevention, green tea catechins have the potential to lower blood cholesterol, a major risk factor for these diseases, by reducing dietary cholesterol and fat absorption from the gastrointestinal tract (Yang and Koo, 2004) as well as by decreasing cholesterol synthesis and increasing the expression of the LDL receptor which is involved in clearing cholesterol from the blood (Bursill and Roach, 2001; Bursill and Roach, 2006). The major tea catechins in green tea are (-)-epigallocatechin 3-gallate (EGCG), (-)- epigallocatecatechin (EGC), (-)- epicatechin (EC) and (-)- epicatechin 3-gallate (ECG). These compounds can account for up to 30% of the dry weight but their relative composition in green tea varies with species, season, age of the leaf (plucking position), climate and horticultural practices (Lin *et al.*, 1996). Epigallocatechin gallate (EGCG) is one of the most remarkable catechins (as well as green tea's most abundant kind of catechin) because it has efficient radical scavenging abilities. However, some studies showed that EGCG and other catechins were unstable under high temperature and neutral or alkaline conditions (pH > 6) (Zhu *et al*., 1997; Chen *et al*., 1998). Therefore, selection of appropriate product to fortify EGCG is important.

Sorbet or sherbet, as it is also called, is frozen foam made from water, nutritive sweeteners, fruit or fruit flavouring, fruit acid, milk solids or egg white, stabilizer and colouring. A sorbet is an acidic food and normally characterized by a fruit or berry flavour (Marshall and Goff, 2003). In general, sorbets are less energy dense than ice cream due to its low fat content. It also tends to be rich in vitamin C because of the fruit or berry content, especially if it contains strawberries (Marshall and Goff, 2003). Therefore, based on its nutritional value, adding EGCG in a sorbet containing strawberries is most likely a better choice than adding it to ice cream.

Materials and Methods

Solvents and standards

The HPLC grade methanol used for the extraction of tea sample were purchased from Merck (Kilsyth, Vic, Australia). Solvents and organic modifiers used in the HPLC mobile phases were acetronitrile, ortho-phosphoric acid and tetrahydrofuran, which were of HPLC grade from Sigma (Castle Hill, NSW, Australia) and AJAX Finechem (Baulkham Hill, Australia), respectively. Milli-Q de-ionized water

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was prepared daily using a Millipore purification system (Millipore Australia PTY, Ltd. North Ryde, NSW, Australia). EGCG (TEAVIGO) that were used as external standards and an ingredient was purchased from DSM, Australia. Purity of the standards were greater than 98%. The internal standard of 4-amino salicylic acid was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Making a strawberry sorbet with EGCG

Fresh strawberries from supermarkets were washed, the stems were cut off and the berries were homogenized to a puree using a Kambrook Bowl Blender. The pureed strawberries and all the other ingredients including EGCG (0.15, 0.20, 0.25 and 0.30%) were measured and mixed in a glass bowl using a hand-held Sunbeam Kitchen Maestro Blender. After measuring the pH of the mixture, it was placed into the chamber of a Breville Gourmet IL Gelatino 1600 Ice Cream Maker which was operated for 25 min. The strawberry sorbets thus produced were then stored at -18ºC (0, 2, 4, 8, 12 and 16 weeks after made) before the extraction.

Extraction of EGCG from a strawberry sorbet

After storage at the indicated times (0, 2, 4, 8, 12 and 16 weeks after made) the samples were melted at room temperature and 50 g samples were extracted in a water bath at 50ºC for 1 hour with 200 ml of methanol and 250 µl of internal standard (I.S. = 0.5 mM 4-aminosalicylic acid) solution was added. After 1 hour, the samples were filtered through a Whatman No. 1 filter paper and an Alltech 0.45 µm nylon syringe filter before they were injected onto the HPLC.

Stability of EGCG in strawberry sorbets under simulated intestinal digestion

To assess the stability of EGCG under simulated intestinal digestion, samples (50 ml), containing either 0.15% or 0.30% (w/v) EGCG in distilled water or 0.15% or 0.30% (w/v) EGCG in strawberry sorbets were adjusted the pH to 7.5 using 40% (w/v) NaOH and then, they were incubated at 37ºC in a water bath for 1 to 12 hours. Samples were taken out of the 37ºC water bath every hour (Figures 2 and 3) and the degradation of EGCG was stopped by adding 3 ml of 0.1 N HCl. After adding I.S. (0.5 mM 4-aminosalicylic acid), the EGCG was extracted and measured as described above.

The HPLC system

The method for determination of amount of Catechins and Caffeine in green tea was developed from a study carried out by Yoshida, Kiso and Goto, 1999. A Shimadzu HPLC system (Shimadzu Scientific Instruments (Oceania) Pty. Ltd, Rydlmere, NSW, Australia) consisted of a computer controlled system with VP 5.03 software, SCL-10A VP system controller, GT-154 degasser, FCV-10AL Mixer, LC-10AD liquid chromatography pump, auto injection SIL-10AXL VP with a 20 µl loop, SPD-10A UV-VIS detector, CTO-10Avp column oven and CBM-10A communications BUS module was used. The chromatographic separation was performed on a C_{18} reversed-phase column. Mobile phase A consisted of 0.2% (v/v) phosphoric acid 86% (v/v), acetonitrile 12% (v/v) and tetrahydofuran 1.5% (v/v) and the mobile phase B consisted of 0.2% phosphoric acid 73.5% (v/v), acetonitrile 25% (v/v) and tetrahydrofuran 1.5% (v/v). The flow-rate of the mobile phase was 1 ml/min. The gradient elution were mobile phase compositions of 100% mobile phase A during the first 30 min, mobile phase B increased from 0% to 100% in the next 10 min, 100% mobile phase B for 20 min, mobile phase B decreased from 100% to 0% for 10 min, and finally mobile phase A for 20 min before the next injection.

Data analysis

Each extraction or procedure was done in triplicate and the values express as the mean (mg of compound per gram of green tea) + the standard error (SEM) for each triplicate. A linear regression analysis of the external standard curves was carried out on Excel 6.0. (Microsoft Office, USA). The SPSS 9 program (SPSS Inc., IL, USA) was used to compare the mean level of the components in the tea infusion using One-way ANOVA and the Post-Hoc test Bonferroni test with statistical significance $P<0.05$.

Results

EGCG was slightly brown colour, no smell and have bitter taste. EGCG did not affect colour and texture of the strawberry sorbet but it affected taste of the strawberry sorbet as the strawberry sorbet has a little bit bitter after taste. Methanol was an effective reagent to extract EGCG as in the experiment, although EGCG was not stable itself after extraction, therefore 3 ml of 0.1 N HCl was added to stop the degradation. Figure 1 shows the chromatograms of EGCG and internal standard in a strawberry sorbet. The HPLC analysis showed that EGCG was very stable in the strawberry sorbet as they remained containing 95 to 97% (Table 1), and there were no significant differences between formulas (0.15, 0.20, 0.25 and 0.30%) at $p< 0.05$. The study of stability

of EGCG in the strawberry sorbet after storage at -18ºC for up to 16 showed that EGCG was relatively stable in the strawberry sorbet at - 18°C for 16 weeks (Table 2) i.e. 81 – 82% and 91 – 92% for the formulas containing 0.15 and 0.30% EGCG, respectively. There was significant difference between week 0 and week 4 in the both formulas but there were no significant differences between week 4 to week 16 in the both formula ($p< 0.05$).

Table 1. Remaining (%) of EGCG from strawberry sorbets immediately after they were made

Percent of EGCG in the formula (w/w)	Remaining (%) of EGCG in the sorbets
0.15 %	95.4 ± 0.4
0.20 %	95.1 ± 1.2
0.25 %	97.4 ± 1.6
0.30 %	97.2 ± 0.8

* Values are means + SD of triplicate samples and there were no significant differences ($p>0.05$)

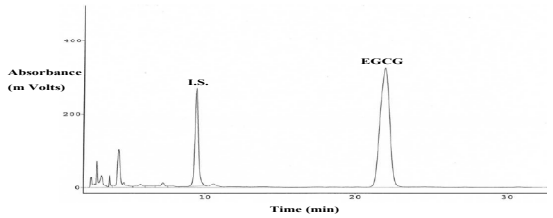

Figure 1. Chromatogram of EGCG and I.S. (4-Aminosalicylic acid) after extraction of a strawberry sorbet containing 0.30% (w/w) EGCG.

The study of stability of EGCG in the strawberry sorbet compare with stability of EGCG in distilled water under simulated intestinal digestion showed that EGCG in the strawberry sorbets (0.30 and 0.15%) were far more stable than EGCG in distilled water. There were significant differences ($p<0.05$) between all samples (Figure 2 and Figure 3).

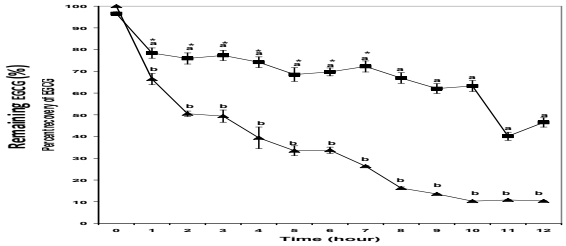

Figure 2. Remaining (%) of EGCG from a strawberry sorbet (■) or distilled water (▲) containing 0.15% (w/w) EGCG after incubation at 37ºC and pH 7.5 for the indicated time. The (a) indicates a significant difference from distilled water and values not sharing (b). (*) indicates a significant difference from each other in the same sample ($P<0.05$).

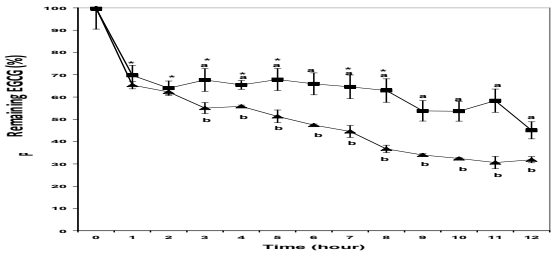

Figure 3. Remaining (%) of EGCG from a strawberry sorbet (■) or distilled water (▲) containing 0.30% (w/w) EGCG after incubation at 37ºC and pH 7.5 for the indicated time. The (a) indicates a significant difference from distilled water and values not sharing (b). (*) indicates a significant difference from each other in the same sample ($p<0.05$).

Discussion

In this study, a strawberry sorbet formula was selected to examine the suitability of this product for adding EGCG. EGCG was hypothesized to remain stable in this sorbet formula because it has a low pH, no heat treatment is involved during its preparation, and it is stored frozen. The upheld hypothesis was that most of the EGCG remained stable and extractable during the processing with a recovery greater than 90% compared to that from freshly made sorbets in different concentrations of EGCG. It was also stable during storage for up to 16 weeks at -18°C. The recovery greater than 90% of EGCG was obtained from the sorbet containing 0.30% w/w EGCG. The EGCG also proved to be more stable in the strawberry sorbet than in distilled water under simulated intestinal digestion conditions.

Stability of EGCG in the fresh (just made) strawberry sorbet

Four strawberry sorbets made with different concentrations of EGCG (0.15, 0.20, 0.25 and 0.30% w/w) were extracted using methanol and analysed by HPLC to measure their EGCG content. This extraction proved to be effective and the HPLC conditions used gave well-defined peaks for EGCG and the internal standard, 4-aminosalicylate, and no other peaks in that area of the chromatogram. Using an external standard curve for the EGCG peak areas normalized to the internal standard peak area, very high recoveries (>90%) of EGCG from the freshly made strawberry sorbets were obtained. The EGCG was therefore very stable in the strawberry sorbets and were easily extracted. The low pH (pH 3.21-3.23) of the strawberry sorbets may have contributed to the stability of the EGCG in this product. The stability of EGCG is well known to be pH dependent – the lower the pH, the greater its stability (Zhu *et al*., 1997; Wang and Zhou, 2004; Chen and *et al*., 2001).

Stability of EGCG in the strawberry sorbet stored at –18°C for up to 16 weeks

The stability of EGCG at levels of 0.15 and 0.30% in the formulas during storage of the strawberry sorbet at –18°C for 16 weeks was also high which is important for the shelf life of the product. However, it appeared that the EGCG was more stable in the sorbet with the highest content of the catechin (0.30%) than in the 0.15% EGCG sorbet. In the 0.30% EGCG sorbet, the lowest recovery was 90.3% while the lowest recovery was 81.6% for the sorbet containing 0.15% EGCG.

Stability of EGCG in the strawberry sorbet under the simulated intestinal digestion

The comparison of the stability of EGCG in the strawberry sorbet with EGCG and EGCG in distilled water under simulated intestine conditions (37ºC and pH = 7.5) showed that the EGCG was more stable in the strawberry sorbet than in distilled water. For example, more than 30% of the EGCG was degraded in the distilled water compared to 20% in sorbet for the first hour of incubation for the sorbet containing 0.15% EGCG. These results also demonstrated that more than 65% of EGCG was recovered from the 0.15% EGCG sorbet after *in vitro* digestion for 10 hours compared to that of less than 10% in distilled water. The 10 hour length is of interest because it represents the time it takes for food to make its way from the mouth all the way through the small intestine. Essentially, the longer the EGCG can remain stable under the neutral pH simulated intestinal digestions the more chance there is that it will be absorbed (Record and Lane, 2001; Chen *et al*., 2001). For the 0.30% EGCG concentration, about 55% of the EGCG in the sorbet remained after 10 hours compared to that of just over 30% for distilled water system. However, it did appear that the EGCG was more stable at 0.30% than at 0.15% in the distilled water on a percentage basis. The high content of vitamin C in strawberries, about 100 mg vitamin C per cup of strawberries, may also have been a factor in the stability of the EGCG in the strawberry sorbet. Vitamin C is a strong antioxidant and may have protected EGCG from degradation in the sorbet. Such a protective effect by vitamin C on EGCG in different aqueous buffer solutions has been reported (Chen *et al*., 2001).

Conclusion

The present results indicate that the strawberry sorbet shows promise as an appropriate product for fortification with EGCG from the point of view of the stability of EGCG in the product and the ease of extraction with methanol and measurement by HPLC. The strawberry sorbet used in this study is also a product with a low energy profile and rich in vitamin C. However, sensory acceptance and physical properties studies need to be conducted on the sorbet in order to determine the feasibility of using it as a new functional food product with added EGCG to lower cholesterol in human.

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