

**Brewing conditions and low-temperature storage affect the total phenolics,  
caffeoylquinic acid content, and antioxidant activity of yerba mate infusion**

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## Abstract

Herbal infusions exhibit diverse pharmacological effects, mainly attributed to the high content of phenolics (e.g., caffeoylquinic acids (CQAs)). Herein, we evaluated the content of CQAs in the methanolic extracts of model herbs, namely yerba mate (*Ilex paraguariensis*), stevia (*Stevia rebaudiana*), and *Pluchea indica*, showing that yerba mate had the highest total CQA content ( $108.05 \pm 1.12$  mg/g of dry weight). The analysis of yerba mate infusions prepared using different steeping times, dried leaf weights, and water temperature demonstrated that the amount of extracted CQAs was maximized ( $\sim 175$  mg per 150 mL) when 6 g of dried leaves were steeped in hot water for 10 min. Ten-day refrigerated storage induced no significant changes in the antioxidant activity and total phenolic and CQA contents of the infusion kept in a brown container but negatively affected the above parameters when kept in a clear container, suggesting the detrimental effect of light exposure. Our study furnishes practical recommendations for improving the preparation and storage of herbal infusions, catering to the needs of consumers, food scientists, and commercial producers. Furthermore, it pioneers an examination into the influence of light exposure on the content of crucial quality attributes (CQA) within plant-based beverages.

**Keywords:** caffeoylquinic acids (CQAs), dried leaf weight, light exposure, refrigerated storage, steeping time, yerba mate infusion

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## Introduction

As naturally occurring plant-derived substances, herbal medicines have long been used to treat numerous diseases and are mainly consumed in the form of herbal teas prepared as infusions (by steeping dried plant parts such as flowers, leaves, seeds, roots, and bark in hot water) or decoctions (by boiling herbs in water).

The health benefits provided by herbal teas are mainly attributed to their high contents of natural bioactive compounds such as phenolics. Caffeoylquinic acids (CQAs) are biologically important phenolics that are derived from hydroxycinnamic acid and exhibit numerous health-beneficial properties such as antioxidant, anti-inflammatory, anticancer, antidiabetic, antihypertensive, and antineurodegenerative activities (Santana-Gálvez et al., 2017). Based on the position, number, and identity of their acyl residues, these compounds, which are naturally produced by plants in response to biotic and abiotic stressors (Farah & Donangelo, 2006), can be categorized into three main groups, namely monocaffeoylquinic acids (monoCQAs: 3-CQA, 4-CQA, and 5-CQA; known as chlorogenic acids), dicaffeoylquinic acids (diCQAs: 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA), and feruloylquinic acids (FQAs: 3-FQA, 4-FQA, and 5-FQA) (Clifford and Wight, 1976; Kremr et al., 2015; Shin et al., 2015).

Previous studies have well documented the presence of CQAs in various herbal infusions. Marques and Farah (2009) identified and quantified CQAs in the infusions of 14 medicinal plants, with the highest CQA contents found in *Ilex paraguariensis* (yerba mate) infusion. Based on the fact that the CQA content of toasted yerba mate was much lower than that of green yerba mate, the authors concluded that CQAs are heat-sensitive. Meinhart et al. (2018) profiled the content of CQAs and caffeic acid in the infusions of 89 plants traditionally used and commercialized in Brazil, revealing that 93% of the tested infusions contained CQAs. The content of CQAs was highest for yerba mate (52.6 mg in 100 mL of infusion). Chewchida and Vongsak (2019) reported the presence of mono- (~2.17 wt% 5-CQA) and diCQAs (~1.24 wt% 3,4-diCQA and ~1.93 wt% 3,5-diCQA) in the infusion of *Pluchea indica* (L.), a traditional Thai medicine widely used owing to its health-promoting properties. Karaköse et al. (2015) profiled the CQA content of stevia (*Stevia rebaudiana* Bertoni), a plant commonly used for sweetening, medicinal, pharmaceutical, and feeding purposes, showing that the corresponding leaf extract contained 5-CQA (~2.69 g/100 g of dry weight (DW)) and 4,5-diCQA (~1.68 g/100 g of DW).

Brewing conditions such as steeping temperature, steeping time, leaf particle size, and water/leaf mass ratio can significantly affect the antioxidant activity and total phenolic content of black and green teas (Khokhar and Magnusdottir, 2002; Komes et al., 2010; Venditti et al., 2010; Yuaan et al., 2015; Hajiaghaalipour et al., 2016; Chang et al., 2020). However, the effects of brewing parameters on the antioxidant activity and phenolic content of herbal infusions remain underexplored and have only been examined by Sentkowska et al. (2016), who investigated the effect of steeping time on the antioxidant activity and phenolic content of chamomile (*Matricaria chamomilla* L.) and St. John's wort (*Hypericum perforatum*) infusions. Considering the numerous health benefits of CQAs, a deeper understanding of the effects brewing conditions have on the CQA profiles of herbal infusions is of utmost importance for consumers and food scientists.

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Another important factor possibly impacting the antioxidant activity and total phenolic content of herbal infusions is low-temperature storage (4 °C). Previous studies have inconsistently reported the effects of low-temperature storage on the quality of teas and infusions (Labbé et al., 2008; Bazinet et al., 2010; Ananingsih et al., 2013; Jiménez-Zamora et al., 2016; Chang et al., 2020). However, to the best of our knowledge, the effect of refrigerated storage on the CQA content of herbal infusions has not been explored. When investigating the effect of storage on CQA content, one should consider temperature and light exposure, as CQAs can be degraded or isomerized at high temperatures or upon irradiation with light (Xue et al., 2016). During storage inside refrigerators, especially those with glass doors, herbal infusions can be periodically exposed to light.

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Herein, we profiled the content of CQAs in the methanolic extracts of model herbs, namely yerba mate, stevia, and *P. indica*, and prepared a homemade infusion of the herb with the highest CQA content (yerba mate) to investigate the effect of brewing conditions and low-temperature storage with and without light exposure on the content of CQAs therein. Thus, our work provides health-conscious consumers with a better understanding of the optimal conditions of herbal infusion preparation and storage while providing a first-time account of the potential effect of light exposure on the CQA content of a plant-based drink.

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## Materials & Methods

### Reagents

Commercial standards (monoCQAs: 1-CQA, 3-CQA, 4-CQA, and 5-CQA; diCQAs: 1,3-diCQA, 1,4-diCQA, 1,5-diCQA, 3,4-diCQA, and 4,5-diCQA) were purchased from Biosynth Carbosynth®, UK (purity ≥ 98.0%), and 3,5-diCQA was purchased from TransMIT GmbH PlantMetaChem (Germany). Puerarin (internal standard) was obtained from Sigma-Aldrich, Inc., St. Louis, MO, USA (purity ≥ 98.0%). Acetonitrile and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). Ultrapure water prepared using the Milli-Q system (Millipore, Billerica, MA, USA) was used in all experiments. All reagents used to determine antioxidant activity and total phenolic content were obtained from Sigma-Aldrich, Inc., St. Louis, MO, USA.

### Plant materials

Plant samples were obtained from reliable commercial sources in Bangkok, Thailand in the form of dried leaves, which is how they are usually consumed. Three brands of each plant were examined. All samples were analyzed without further drying to maintain the original content of CQAs.

### Preparation of methanolic extracts

Samples were ground to a fine powder passing through a 0.75-mm sieve using a mixer mill (MM 400, Retsch GmbH) at 30 Hz for 1 min. A 20-mg aliquot (DW) of each ground sample was extracted with 1 mL of 80 vol% aqueous methanol containing puerarin (0.05 g L<sup>-1</sup>) as an internal standard upon vigorous 15-min shaking at 1500 rpm and 15 °C (Eppendorf Thermomixer® C, Eppendorf, USA). The resulting mixtures were centrifuged at 12000 g and 4 °C for 15 min using a table-top centrifuge (5415 R, Eppendorf, USA), and the supernatants were collected, filtered through a 0.2-µm nylon syringe filter, and injected into a high-performance liquid chromatograph for CQA analysis (Khaksar et al., 2021).

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### Infusion preparation

The effects of brewing conditions on the antioxidant activity and CQA content were investigated for the infusion of the plant with the highest CQA content (yerba mate).

#### Effects of water/leaf mass ratio and steeping time

Dried yerba mate leaves (2 (equivalent to plant mass in a commercial sachet), 4, 6, and 8 g) were added to 150 mL (volume of a medium-size cup) of hot water (~90 °C) and steeped for 5 min (according to most manufacturers). The resulting infusions were filtered through paper (Whatman No. 1), immediately cooled in an ice bath, filtered through a 0.2-µm nylon syringe filter, and analyzed (chromatography, total phenolic content, and antioxidant activity). To further investigate the effect of steeping time (10, 15, and 30 min) on CQA content, the corresponding infusions were filtered and analyzed as described in the related sections. The thus identified optimal brewing conditions were used for subsequent experiments.

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#### Effects of repeated infusion preparation

Many consumers repeat the infusion preparation by adding water to steeped leaves. Accordingly, to examine the effect of this process on CQA content, a yerba mate infusion was prepared using hot water under the optimal conditions obtained in section 2.4.1. After filtering, the infusion preparation was repeated by adding 150 mL of hot water to the steeped leaves (second cycle), and this process was repeated one more time (third cycle). After each cycle, the infusion was filtered through a 0.2-µm nylon syringe filter and analyzed (chromatography, total phenolic content, and antioxidant activity) as described in the related sections.

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#### Effects of steeping temperature

Steeping with cool water is a common practice among yerba mate consumers, especially in Paraguay and Southern Brazil (<https://mateina.ca/pages/preparation>). Accordingly, to reproduce these conditions and investigate the effect of steeping temperature on the CQA content, total phenolic content, and antioxidant activity, we prepared an infusion using cool water (~27 °C) under the optimal conditions obtained in section 2.4.1, subjected it to the corresponding analyses, and compared the results with those obtained for the infusion prepared using hot water.

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#### Effects of low-temperature storage

To investigate the effect of storage under simulated refrigeration conditions on the antioxidant activity, total phenolic content, and total CQA content, we prepared a yerba mate infusion under the optimal conditions obtained in section 2.4.1. The infusion was filtered, processed as described above, and stored inside brown (dark condition) and transparent (normal condition) air-tight glass containers inside a glass-door refrigerator (4 °C) for 10 days. After 1, 3, 5, 7, and 10 days of storage, the samples were filtered through a 0.2-µm nylon syringe filter and analyzed.

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#### Antioxidant activity determination

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was assessed following the method of Brand-Williams et al. (1995) with some modifications (Khaksar et al., 2019). DPPH radical scavenging activity was calculated as:  
$$\text{DPPH radical scavenging activity (\%)} = 100 \times (A - A_1 + A_2)/A,$$

where A (absorbance) is the  $A_{515}$  of the DPPH solution without the infusion,  $A_1$  is the  $A_{515}$  of the DPPH solution with the infusion, and  $A_2$  is the  $A_{515}$  of the infusion.

#### Total phenolic content

Total phenolic content was measured according to the Folin-Ciocalteu method (Swain and Hillis, 1959). Total phenolic acid content was expressed as g gallic acid equivalent (GAE) per 150 mL.

#### Total microbial count determination and color analysis

Total microbial count was determined following the method described in our previous study (Khaksar et al., 2019). The results were expressed as log colony-forming units (CFU) per mL of sample. For color analysis, three color parameters, namely  $L^*$  (lightness),  $a^*$  (redness/greenness), and  $b^*$  (yellowness/blueness), were measured using a colorimeter (CR-410 chroma meter, Minolta, Japan). Color difference ( $\Delta E$ ) was calculated using the freshly prepared infusion as a control:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$$

#### Ultraperformance liquid chromatography (UPLC)

The CQA profiling of infusions was performed using an UltiMate 3000 UPLC system coupled with a Dionex UltiMate DAD 3000 detector (Thermo Fisher Scientific, Waltham, MA, USA) and equipped with a Kinetex EVO C18 column (250 mm  $\times$  4.6 mm, 5  $\mu$ m) (Phenomenex, USA) at a detection wavelength of 325 nm following the method described in our previous study (Khaksar et al., 2021). Peaks matching the retention times and UV spectra of commercial standards were identified as CQAs. Puerarin was used as an internal standard. Each CQA was quantified according to its calibration curve in the range of 1.30–500  $\mu$ g/mL. More information on the employed CQA standards, including standard curves and equations, limit of detection, and limit of quantification, is presented in Supplementary Data (Supplementary Fig. S1 and Supplementary Table S1).

#### Statistical analysis

The acquired data were subjected to statistical analysis using SPSS software v. 20 (SPSS Inc., IBM) and presented as means  $\pm$  standard deviations of three independent biological replicates. Statistical comparisons of means were carried out using one-way ANOVA followed by Duncan's multiple range test or Student's  $t$ -test at the 0.05 confidence level.

## Results

#### CQA profiles of methanolic extracts

Six CQAs (3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) were identified and quantified in the investigated plants (Table 1). The total CQA content decreased in the order of yerba mate (108.05  $\pm$  1.12 mg/g DW basis) > stevia (79.26  $\pm$  0.74 mg/g DW basis) > *P. indica* (64.59  $\pm$  1.18 mg/g DW basis). Notably, whereas yerba mate and stevia contained all six of the above CQAs, *P. indica* contained only five (i.e., did not contain 4-CQA). The contributions of mono- and diCQAs to the total CQA content depended on the plant, i.e., the

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major contributor was identified as monoCQAs for yerba mate and stevia and as diCQAs for *P. indica*. The distribution of monoCQAs also depended on the plant, i.e., 3-CQA was the most abundant monoCQA in yerba mate, while 5-CQA was most abundant in stevia and *P. indica*. Regarding diCQAs, 3,5-diCQA was the major isomer in all samples. Fig. 1 presents the chromatograms of the analytical standards and the yerba mate sample.

For each plant, the observed CQA distributions were similar across different commercial brands. No significant variation in the contents of individual isomers was found for stevia and *P. indica*, whereas for yerba mate, monoCQA isomers were significantly more abundant in brand A than in brands B and C (Table 1), which was ascribed to the effects of cultivation and climatic conditions and/or processing. In addition, we did not observe significant differences in total CQA content between methanolic and aqueous extracts of yerba mate.

## Effects of brewing conditions

### Effects of water/leaf mass ratio and steeping time

For a 5-min steeping time (recommended by most manufacturers), the antioxidant activity (Fig. 2A), total phenolic content (Fig. 2B), and total CQA content (Fig. 2C) of yerba mate infusions increased with increasing dried leaf weight, peaking at a dried leaf weight of 8 g. However, for steeping times of 10, 15, and 30 min, these parameters were maximized at a dried leaf weight of 6 g and did not change when this weight increased to 8 g (Fig. 2). At a constant dried leaf weight, an increase in steeping time from 5 to 10 min significantly increased antioxidant activity, total phenolic content, and total CQA content, whereas a further steeping time extension to 15 and 30 min had no effect (Fig. 2). Thus, the optimum brewing conditions corresponded to a steeping time of 10 min and a dried leaf weight of 6 g.

### Effects of repeated infusion preparation

Repeated infusion preparation negatively affected antioxidant activity (Fig. 3A), total phenolic content (Fig. 3B), and CQA content (Fig. 3C), all of which were minimized after the third cycle. Thus, the infusion with the highest antioxidant ability, total phenolic content, and total CQA content was prepared using a single steeping cycle.

### Effects of steeping temperature

Given that CQAs are heat sensitive, we investigated the effects of steeping temperature on the quality of the resulting infusion. Surprisingly, the antioxidant activity, total phenolic content, and total CQA content of the infusion prepared using cool water were significantly lower than those of the infusion prepared using hot water (Fig. 4).

### Effects of simulated refrigerated storage

#### Effects on antioxidant activity, total phenolic content, and total CQA content

The refrigerated storage and on-demand consumption of home-made infusions is a regular practice among consumers. Herein, the storage of the yerba mate infusion in a brown glass container (dark condition) at 4 °C did not affect its quality, i.e., during 10-day storage, the antioxidant activity (Fig. 5A), total phenolic content (Fig. 5B), and total CQA content (Fig. 5C) did not significantly deviate from those of the control sample (freshly prepared infusion). However, low-temperature storage negatively affected infusion quality when a transparent glass

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container (normal conditions) was used. As shown in Figs. 5A, 5B, and 5C, all measured parameters remained unchanged until day seven, significantly decreasing on day 10.

#### Effects on total microbial count and color

Ten-day storage at 4 °C did not affect the total microbial count, i.e., during the storage period, the counts of total aerobic bacteria (Supplementary Fig. S2A) and yeast/mold (Supplementary Fig. S2B) did not significantly deviate from those of the control sample. Similarly, no significant changes were observed for  $L^*$ ,  $a^*$ , and  $b^*$  (Supplementary Table S2). The color difference ( $\Delta E$ ) did not significantly vary during storage and remained within the range of 0–1, suggesting an invisible difference.

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## Discussion

Yerba mate, widely consumed in South America, is well known for its high content of phenolics such as CQAs. Herein, six CQA isomers (three monoCQAs and three diCQAs) were identified and quantified in this plant. MonoCQAs accounted for 60% (on average) of the total CQA content and were mainly represented by 3-CQA, while diCQAs were mainly represented by 3,5-diCQA. Thus, our findings are in line with those of Clifford and Ramirez-Martinez (1990), Marques and Farah (2009), and Meinhart et al. (2018). Notably, Marques and Farah (2009) reported the presence of caffeine in the methanolic extract of yerba mate (~15 mg per 100 g of DW), which is something caffeine-sensitive consumers should be aware of.

The methanolic and aqueous extracts of yerba mate had similar total CQA contents, in line with the report of Marques and Farah (2009), which suggested that a satisfactory CQA extraction efficiency is achieved during the home preparation of yerba mate infusions. Data on the CQA content of beverages (except for coffee, which is well known to be rich in CQAs) are scarce. Jeon et al. (2019) determined the total content of CQAs in instant coffee (100%), ready-to-drink coffee (non-milk added coffee), and ground coffee (unblended and roasted) as ~80.7, 75.02, and 217 mg per 150 mL, respectively. Greenrod et al. (2005) determined the total phenolic content of red wine (which is a good source of phenolics) as ~100 mg per 150 mL. The CQA-rich yerba mate commonly consumed as a homemade infusion deserves attention in view of the pharmacological activity of CQAs. However, one cannot claim that all plants with high CQA contents are good sources of CQAs for humans, as the influence of the food matrix on CQA bioavailability is still unknown. Moreover, it would not be correct to assert that the lower CQA contents of some herbal infusions make them not important to humans, as the metabolism and requirements of these compounds seem to vary amongst individuals, with no dietary recommendations currently established.

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During repeated steeping, CQA content was minimized at the end of the third cycle (~ 75 mg per 150 mL) (Fig. 3) but was still comparable to that of instant and ready-to-drink coffee, which suggested that satisfactory CQA extraction was achieved even upon repeated infusion preparation. Altogether, ~375 mg of CQAs (~58% of the total content in 6 g) was extracted over three cycles.

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Herbal infusion preparation using cool water is a common practice among consumers, e.g., infusions prepared by 2-h steeping at ~25 °C have become popular in Taiwan (Venditti et al., 2010). Herein, the antioxidant activity, total phenolic content, and total CQA content of the cold-brewed infusion were significantly lower than those of the hot-brewed infusion (Fig. 4), in agreement with the results previously obtained for the total phenolic content and antioxidant activity of white, green, and black tea infusions prepared with cold water (Khokhar and

Magnusdottir, 2002; Venditti et al., 2010; Yuann et al., 2015; Hajiaghaalipour et al., 2016). The effect of steeping temperature on CQA content observed herein was attributed to the increased solubility of CQAs in warm water (De Maria et al., 1998). Moreover, Dibert et al. (1989) reported that the diffusivity of chlorogenic acids in water increases with increasing temperature. A rise in plant cell permeability with increasing temperature may also increase the transference rate of CQAs. Notably, the preparation of the yerba mate infusion under optimum brewing conditions (steeping of 6 g of dried leaves for 10 min) using hot water did not have any detrimental effect on CQA content.

Although the refrigerated storage and on-demand consumption of homemade infusions is a regular practice, health-conscious consumers are often concerned about the effects of storage conditions on the nutritional qualities of infusions. Therefore, we investigated the effect of refrigerated storage on the antioxidant activity, total phenolic content, and CQA content of a homemade yerba mate infusion over a 10-day period, revealing that these parameters were not affected in the case when storage was performed in a brown glass container but experienced deterioration when storage was performed in a transparent glass container (Fig. 5). Thus, our findings strongly suggest that exposure to light has a detrimental effect on the antioxidant activity, total phenolic content, and total CQA content of infusions. To the best of our knowledge, this is the first report on the effect of light on the CQA content of a drink.

Color stability is an important quality characteristic of drinks during storage. Unlike in the case of CQA content, exposure to light did not affect the color stability and total microbial count of the infusion during storage.

## Conclusions

~~In summary, we profiled~~ the content of CQAs in the methanolic extracts of three medicinal plants, namely yerba mate, stevia, and *P. indica*, showing that yerba mate had the highest total CQA content. Subsequently, we prepared a homemade infusion of yerba mate and investigated the effects of brewing conditions (steeping time and temperature, leaf/water mass ratio, and repetitive steeping) and refrigerated storage (4 °C, light and dark conditions) on its antioxidant activity, total phenolic content, and total CQA content. Except for low-temperature storage under dark conditions, which did not have any detrimental effect, all factors significantly affected antioxidant activity, total phenolic content, and CQA content. Considering the numerous health benefits of CQAs, our findings provide health-conscious consumers and food scientists with an improved understanding of the optimal conditions for the preparation and storage of herbal infusions.

## Acknowledgments

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## Figure legends

**Fig. 1.** Chromatograms of (A) the analytical standards and (B) the yerba mate sample.

**Fig. 2.** Effects of the water/leaf mass ratio and steeping time on the (A) antioxidant activity, (B) total phenolic content, and (C) total CQA content of yerba mate infusions. Error bars represent means  $\pm$  standard deviations ( $n = 3$ ). For each steeping time, comparisons are made among different leaf weights; bars with different letters differ significantly according to Duncan's multiple range test ( $p < 0.05$ ).

**Fig. 3.** Effect of repeated preparation on the (A) antioxidant activity, (B) total phenolic content, and (C) total CQA content of yerba mate infusions. Error bars represent means  $\pm$  standard deviations ( $n = 3$ ). For each parameter, comparisons are made among three cycles; bars with different letters differ significantly according to Duncan's multiple range test ( $p < 0.05$ ).

**Fig. 4.** Effect of steeping temperature (90 and 27 °C) on the (A) antioxidant activity, (B) total phenolic content, and (C) total CQA content of yerba mate infusions. Error bars represent means  $\pm$  standard deviations ( $n = 3$ ). For each parameter, comparisons are made between infusions prepared using hot and cold water; bars with different letters differ significantly according to Student's *t*-test ( $p < 0.05$ ).

**Fig. 5.** Effect of low-temperature storage on the (A) antioxidant activity, (B) total phenolic content, and (C) total CQA content of yerba mate infusions. Error bars represent means  $\pm$  standard deviations ( $n = 3$ ). For each day, an asterisk (\*) above the bars indicates a significant difference compared to the sample at day 0 (control) (Student's *t*-test,  $p < 0.05$ ).

