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Polyphenol input to the antioxidant activity of yerba mate (*Ilex paraguariensis*) extracts

Julia Valerga [a], Mario Reta [b], Maria Cecilia Lanari [a,*]

[a] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET - La Plata, Facultad de Ciencias Exactas Universidad Nacional de La Plata (UNLP), Calle 47 y 116 S/N°, La Plata (B1900AJJ), Buenos Aires, Argentina
[b] Departamento de Química Analítica, Facultad de Ciencias Exactas (UNLP), Argentina

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ABSTRACT

Industrial processing modified the polyphenol content, composition and antioxidant activity of the yerba mate extracts. Pre-dried leaves were the most appropriate raw material combining maximum activity with high polyphenol content. Chlorogenic acid and its derivatives were the major components of the phenolic fraction but we also identified caffeic, rutin and quercetin.

Yerba mate extracts inhibited malonedialdehyde formation in sunflower oil (20 μmol/kg) and conjugated dienes production in oil/water emulsions (60 μmol/kg). Enhancing the dose to 60 μmol/kg reduced 27.8% the extract's activity in oil.

The relationship between polyphenol composition and antioxidant activity of a mixture of caffeic, chlorogenic, kaempferol, quercetin and rutin was satisfactorily predicted with a polynomial model. Results showed that quercetin was the highest contributor to the linear term followed by kaempferol and caffeic acid while rutin and chlorogenic acid inputs were the lowest. The model detected five synergistic and six antagonistic effects.

1. Introduction

Yerba mate (*Ilex paraguariensis*) tea, a widely consumed beverage in Argentina, Brazil, Paraguay and Uruguay, is gaining rapid penetration into the USA and the European countries because of its alleged therapeutic capacity. Several publications reported that yerba mate tea has hypocholesterolemic, hepatoprotective, diuretic and antioxidant properties (Filip & Ferraro, 2003; Filip, Lottito, Ferraro, & Fraga, 2000). It can also improve the cardiovascular system (Schinella, Troiani, Davila, de Buschiazzo, & Tournier, 2005) and act as a central nervous system stimulant (Gonzales, Ferreira, Vazquez, Moyna, & Paz, 1993). Mate infusions have been proposed as a dietary supplement for the prevention of arteriosclerosis and coronary heart disease (Carini, Facino, Aldini, Calloni, & Colomo, 1998; Heck & González de Mejia, 2007). In a recent publication, Puangpraphant and Gonzalez de Mejia (2009) demonstrated that yerba mate phytochemicals inhibited pro-inflammatory markers. Some of the pharmacological properties attributed to mate tea have been related to its high content of polyphenolic antioxidants especially chlorogenic acid (CL) and its derivatives (3, 4-di-O- dicaffeoylquinic, 3, 5-di-O- dicaffeoylquinic and 4, 5-di-O-dicaffeoylquinic acids), caffeic acid (C) as well as flavonoids like quercetin (Q), rutin (R) and kaempferol (K) (Heck, Schmalko & Gonzalez de Mejia, 2008).

Oxidative rancidity is the main cause for the development of undesirable changes in flavor, color and the production of toxic carcinogenic compounds in food products. Research published in the last years and subsequent publicity has fostered significant consumer resistance to the use of synthetic antioxidants and a growing interest for "natural" minimally processed additives that can extend the shelf life of both processed and unprocessed food. A recent report showed that beside its positive health properties, yerba mate antioxidant extracts inhibited lipid oxidation in salami (Campos et al., 2007). Therefore we could infer that incorporation of polyphenolic extracts of yerba mate to food could be a simple and effective way of improving their nutritional and sensory quality as well as extending their shelf life.

Industrial production of yerba mate involves a blanching step or "zapecado" followed by pre-drying and drying. During the zapecado, the green leaves are exposed to direct fire for 20–40 s, reaching temperatures between 120 and 140 °C. The "zapecada" leaves are pre-dried 3–5 min with hot air at 80–100 °C and then dried with a continuous belt system at 90–110 °C for 2–3 h until the leaves reach 2–5% relative humidity. Once dried the leaves

* Corresponding author. Tel.: +54 221 424 9287/425 4853.
E-mail address: clanari@cidca.org.ar (M.C. Lanari).

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Nomenclature

AI	antioxidant index
AOA	antioxidant activity
C	caffeic acid
CL	chlorogenic or chlorogenic acid
DCY	dried/canchada" yerba mate
DL	dried leaves
FAY	forced aged yerba mate
GAE	gallic acid equivalents
GL	yerba mate fresh green leaves
hp	hydroperoxide
HPLC	high performance liquid chromatography
K	kaempferol
Q	quercetin
PDY	pre-dried yerba mate
R	rutin
TP	total polyphenol
ZY	zapecada yerba mate

were coarsely ground to facilitate handling in a process called "canchado" and subjected to natural or forced aging. Natural aging conditions include keeping the canchada leaves for 9–12 months at natural temperature/humidity conditions; in contrast, forced aged yerba is stored at 69 °C and 49% humidity for 62 days. A recent report (Isolabella et al., 2010) showed that industrial processing significantly altered the composition of bioactive compounds. Samples from zapecada, dried or aged yerba mate contained a higher content of bioactive compounds than the green leaves (Isolabella et al., 2010) and consequently may have higher antioxidant activity.

It is well established that the antioxidant activity of a mixture depends on the identity and concentration of the active components (Evans, 1997). Although a positive relationship between antioxidant activity and total polyphenol level of *I. paraguariensis* extracts has been demonstrated (Heck et al., 2008), the individual contributions and the potential interactions between the mixture components has not been determined.

The objectives of this study were:

a) To analyze the effect of industrial processing on the antioxidant activity of polyphenolic extracts from yerba mate.
b) To determine the efficiency of the extracts to reduce oxidative rancidity on sunflower oil free of added antioxidants and oil/water emulsions.
c) To determine the relationship between the composition of a mixture of selected polyphenols commonly found in yerba mate and its antioxidant activity in emulsified linoleic acid.

2. Materials and methods

2.1. Extracts

Samples from fresh green leaves (GL) and from blanched or "zapecada" (ZY), pre-dried (PDY). "dried/canchada" (DCY) and forced aged (FAY) yerba mate (*I. paraguariensis*. St Hil) were obtained from Est. Las Marias SAIC (Gov. Virasoro, Corrientes ARGENTINA). All samples were harvested the same day in May and belonged to the same plot to avoid variability.

Immediately after the arrival to the laboratory (48 h after harvest), the fresh green leaves were frozen by immersion in liquid nitrogen and stored at −80 °C until required for analysis. The ZY, PDY and DCY samples were kept at 4 °C.

After storage, the samples were ground to fine powder in a coffee mill (Moulinex Corp., Buenos Aires ARG.) and extracted at least 5 times with acetone/H_2O 80/20 using a Soxhlet extractor (Turkmen, Sari, & Velioglu, 2006). The extracts were concentrated under reduced pressure with a rotary evaporator at 40 °C. All determinations were made with freshly extracted samples.

2.2. Poyphenol content and composition

The total phenolic content of the extracts (TP; mg of gallic acid equivalents (GAE)/g dried leaves (DL)) was determined by the Folin–Ciocalteau method (Singleton & Rossi, 1965).

The chromatographic analysis of the studied polyphenols was done with an HP 1100 liquid chromatograph, equipped with a binary pump, thermostatized column compartment, auto injector, degasser and diode array detector connected to an HP workstation. The column was a Zorbax 300 SB-C18 (250 × 4.6 mm, i.d.) packed with 5 μm particles and connected to a guard column.

Chromatographic separations were carried out using a mixture of water, methanol and formic acid (79.7:20:0.3) (A) and a solution of methanol and formic acid (99.7:0.3) (B) as mobile phase with a flow rate of 0.9 ml/min. The gradient program was: 0–15 min pure A; 15–30 min A/B 90/10; 30–40 min A/B 70/30; 40–50 min 4A/B 40/60; 50–52 min A/B 20/80; 52–57 min pure A (Gonzalez de Mejia, Young, Heck, & Ramirez-Mares, 2010)

The identification and quantification of the polyphenols was done by comparing their retention times and DAD spectra with those from five analytical grade (≥98%) commercial standards: caffeic (C), chlorogenic (CL), kaempferol (K), quercetin (Q) and rutin (R) (Sigma–Aldrich Buenos Aires, Argentina). TP and extract composition determinations were replicated at least 3 times and the polyphenol concentrations were expressed in mg/g DL.

2.3. Effect of industrial processing on the antioxidant activity of yerba mate extracts

The capacity of freshly prepared yerba mate extracts to inhibit β-carotene bleaching in a β-carotene-linoleic acid emulsion was monitored using the protocol reported by Wettasinghe and Shahidi (1999) with slight modifications. The β-carotene solution was prepared by dissolving 2 mg of β-carotene (Sigma–Aldrich Buenos Aires, Argentina) in 10 ml chloroform. 2 ml of this solution were mixed with 400 mg of Tween 40 (Sigma–Aldrich Buenos Aires, Argentina). After chloroform removal, 40 mg of linoleic acid were added and the total volume was adjusted to 100 ml with water. Aliquotes (4.8 ml) of this emulsion were transferred to tubes and mixed with 0.2 ml of the antioxidant solution. The concentration of antioxidant used in this step was adjusted in order to have a TP final level of 20 μmol GAE/l.

The zero time absorbance was measured immediately after adding the emulsion. After heating the tubes at 50 °C for 120 min to initiate peroxidation, the absorbance was recorded at 470 nm. Blank samples free of added carotene were prepared in a similar way for background subtraction. The remaining β-carotene concentration in the reaction medium was calculated with a calibration curve made with gallic acid and expressed as mg GAE/ml extract. The antioxidant index (AI) was calculated using Eq. (1).

$$AI = 100^{*}(\beta - \text{carotene})_{120}/(\beta - \text{carotene})_{0} \quad (1)$$

$(\beta\text{-carotene})_0$ and $(\beta\text{-carotene})_{120}$ are the β-carotene concentrations at reaction time = 0 and after 120 min.

2.4. Antioxidant activity of the yerba extracts on bulk oil and oil/water emulsions

The polyphenol extract used in these studies was obtained from freshly extracted DCY samples. The volume of extract was adjusted in order to have a TP final concentration of 20 (Y20) or 60 (Y60) μmol GAE/kg sample.

2.4.1. Bulk sunflower oil free of added antioxidants

1 ml of a methanolic solution of the extract was added to 50 g of oil, and stored in 250 ml glass bottles at 60 °C for 32 days. The control samples had 1 ml of methanol with no antioxidant. The reference was a commercial extract with 50% natural tocopherols (20 μmol/kg oil; Toc20). Lipid oxidation was monitored with the TBARS method (AOCS, 2009) at days 0, 4, 8, 16, 25 and 32; results were expressed in mg malondialdehyde (MDA)/kg oil.

2.4.2. Oil in water emulsions

A mixture of sunflower oil (10%), Tween 20 (1%; Sigma–Aldrich, Buenos Aires, ARG) and water (89%) were homogenized with the antioxidant extracts for 5 min using a Braun MR (Buenos Aires ARG) mixer. The sunflower oil employed in this assay belonged to the same batch as the one used in Section 2.4.1. The resulting emulsions were transferred to 500 ml capped glass bottles and stored at 60 °C for 2 days. Variations in the conjugated dienes content (mg hydroperoxides (hp)/g emulsion (em)) were utilized as a measure of antioxidant activity (Pazos, Gallardo, Torres, & Medina, 2005).

2.5. Relationship between polyphenol composition and antioxidant activity

The relationship between polyphenol composition and antioxidant activity was determined using different combinations of caffeic (C), chlorogenic (CL), quercetin (Q), rutin (R) and kaempferol (K) (Sigma–Aldrich, Buenos Aires, ARG). The total polyphenol concentration of the blend was 20 μmol/l. Antioxidant activity was measured with the β-carotene-linoleic acid method (Wettasinghe & Shahidi, 1999) described in Section 2.3.

2.6. Statistical analysis

The effect of industrial processing on the TP content and on the antioxidant activity of the yerba mate extracts was analyzed using the SYSTAT software (1998). Significant differences among means were determined by analysis of variance followed by pairwise comparisons with the Student 't" test. P values < 0.05 were considered statistically significant. Results were expressed as the mean ± standard deviation (SD) of at least 3 replicates.

The experimental design adopted to determine the relationship between the polyphenol composition and antioxidant activity consisted of a simplex lattice mixture design (Cornell, 1990) replicated 3 times, with 5 factors (C, CL, K, Q, R) at 4 levels each, The design was generated by the Systat 12 software (Systat, 2007) and resulted in a total of 105 runs.

The analysis was performed with the following model: n n

$$AI_{pred} = \sum_{i=1}^{n} \beta i\, xi + \sum_{i<j=1}^{n} \sum \beta ij\, xi\, xj \quad (2)$$

Where AI_{pred} represents the AI values predicted by the model; βi and βij are the coefficients for the linear and interaction terms respectively. Positive and negative values of βij indicate synergistic and antagonistic effects respectively (Cornell, 1990). Treatment effects and estimates of the model parameters were determined using the General Linear Model and Regression Procedures from Systat (2007). To eliminate unnecessary terms from the model, within each group of effects (linear, 2nd or 3rd way interactions) we determined the differences ($P < 0.05$) between coefficients; similar coefficients ($P > 0.05$) were grouped and replaced by their average (Cornell, 1990).

Model validation was carried out using combinations of the variables at different levels within the experimental range.

3. Results and discussion

3.1. Effect of industrial processing on polyphenol content and composition and antioxidant activity of the yerba mate extracts

3.1.1. Total polyphenol content and phenolic composition of the extracts

Table 1 presents the effect of "zapecado", pre-drying, ¨drying/canchado" and forced aging on the TP_{FC} content of the extracts determined with the method of Folin-Ciocalteau. The zapecado was the only process that significantly affected ($P < 0.05$) the total polyphenol contents of the leaves; the TP_{FC} level of the ZY samples was 22 times higher than the one from the fresh leaves. In accordance with previous publications (Holovatty, Argüello, & Malec, 2006; Isolabella et al., 2010), no significant differences ($P > 0.05$) were detected upon further processing and storage.

The TP_{FC} contents in the FAY samples were 15% lower than those informed by Turkmen et al. (2006) working with retail products. These differences could be due to variations in the raw material, processing and/or storage conditions.

HPLC analysis indicated that the extracts from GL samples (Fig. 1A) contained chlorogenic (tr = 11 min) and caffeic (tr = 13 min) acids, rutin (tr = 37 min) and quercetin (tr = 42 min) while the DCY leaves had CL and C (Fig. 1B). Phenolic profiles from the ZY extracts indicated the presence of CL and R while those corresponding to the PDY and FAY samples had CL and C (Data not shown). None of the samples contained kaempferol.

All the phenolic profiles showed that for retention times less than 15 min and longer than 25 min, several peaks with a DAD spectrum very similar to the one from the chlorogenic acid suggesting that they may correspond to chlorogenic acid derivatives

Table 1
Effect of the processing step on the total polyphenol content and composition of the yerba mate extracts.

Processing step	TP^{*}_{FC}	SD	TP^{*}_{HPLC}	SD	C^{*}	SD	CL^{*}	SD	R^{*}	SD	Q^{*}	SD	DCL^{*}	SD
Fresh leaves	4.15^{a}	0.14	5.93^{a}	0.05	0.10^{a}	8×10^{-3}	0.08^{a}	8×10^{-3}	3.61^{a}	0.4×10^{-1}	0.55	5.7×10^{-2}	1.59^{a}	0.013
Zapecado	98.86^{b}	12.51	60.64^{b}	6.03	nd	nd	6.92^{b}	0.07	15.49^{b}	1.44	nd	nd	38.24^{b}	3.66
Pre-drying	90.23^{b}	4.08	38.02^{c}	3.77	0.09^{a}	8×10^{-3}	14.5^{c}	1.32	nd	nd	nd	nd	23.43^{c}	2.44
Drying/canchado	96.07^{b}	5.77	33.69^{c}	3.44	0.16^{b}	1.33	13.01^{c}	1.47	nd	nd	nd	nd	20.51^{c}	2.13
Forced aging	101.00^{b}	2.39	70.01^{b}	6.98	7.3^{c}	0.71	1.13^{a}	0.12	nd	nd	nd	nd	62.49^{d}	6.45

TP_{FC}: total polyphenol (TP) content determined by Folin-Ciocalteau assay; TP_{HPLC}: total polyphenol (TP) content determined by HPLC.
Asterisk denote concentrations in mg/g d DL (dried leaves); SD: standard deviation; Caffeic (C); Chlorogenic (CL); Quercetin (Q); Rutin (R) and DCL chlorogenic derivatives.
$^{a-d}$Means within each column having the same letter are not significantly different ($P > 0.05$).

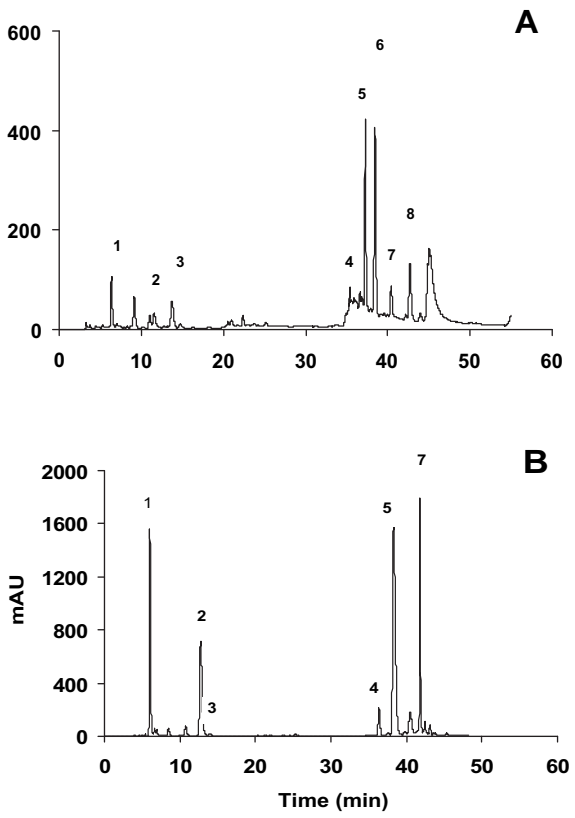

Fig. 1. HPLC chromatogram of the extracts from fresh (A) and dried/"canchada" (B) samples. (2) chlorogenic acid; (3) caffeic acid; (6) rutin and (8) quercetin. Peaks 1,4,5,7 were tentatively identified as neochlorogenic acid, 3,4-, 3,5- and 4,5-dicaffeoyl quinic acids respectively. 330 nm —; 360 nm - - - .

(CLD). Gonzáles de Mejía et al., (2010), using the same experimental conditions applied in the current study, reported that chlorogenic acid isomers like the 3- and 4-caffeoylquinic acids had retention times values ranging from 5 to 16 min. Dicaffeoylquinic compounds like the 3,4; 3,5 and 4,5 dicaffeoylquinic acids were detected after tr = 30 min (Gonzáles de Mejía et al., 2010). Because of the lack of commercial standards, a tentative identification of the mono and the caffeoylquinic isomers was done based their elution profiles determined with a similar column (Bravo, Goya, & Lecumberri, 2007; Gonzáles de Mejía et al. 2010). Peaks 1, 4, 5 and 7 (Fig. 1A and B) may correspond to neochlorogenic acid, 3,4-, 3,5- and 4,5-dicaffeoyl quinic acids respectively.

Total mono- and dicaffeoylquinic isomers (CLD) concentration was calculated using chlorogenic acid as a standard. A similar procedure was followed to identify and quantify the chlorogenic acid derivatives in the ZY, PDY and FAY samples (Data not shown).

Table 1 shows the influence of industrial processing on the phenolic profile of the extracts. In accordance with Chandra and Gonzalez de Mejia (2004) and Bravo et al. (2007), the chlorogenic acid and its derivatives were the major components of the phenolic fraction with concentrations ranging from 28% to 99%. Chlorogenic acid content increased with zapecado from 0.08 to 6.92 mg/g DL, a subsequent pre-drying treatment duplicated the amount present in the ZY extracts. No changes were observed in the DZ samples however, forced aging had a very strong effect since it reduced CL concentration by 92%. Industrial processing enhanced CLD total content (calculated as the sum of the individual levels) from 1.59 to 62.49 mg/g DL. Isolabella et al. (2010) also reported that extracts from zapecado, dried and aged leaves had higher concentrations of caffeoyl derivatives than those obtained from freshly harvested leaves, however, our results showed a much stronger impact of the industrial process. These differences could be attributed to variations in the sample handling procedure between both studies. Ferracane et al. (2008) showed that blanching, steaming or frying enhanced artichokes chlorogenic acid derivatives concentration between 66 and 94%. In their publication, Isolabella et al. (2010) dried the fresh yerba mate leaves at 40 °C before analysis, which may cause an increment in the CL and CLD content (Ferracane et al., 2008) resulting in a reduction of the processing effect.

Zapecado and pre-drying did not affect caffeic acid level; nevertheless, drying and specially forced aging caused a considerable increment. Rutin was only detected in the FY and ZY samples.

A comparison between the TP contents determined with the Folin-Ciocalteau (TP_{FC}) and the HPLC (TP_{HPLC}) indicated that the polyphenol identification have been more successful in the GL, ZY and FAY samples where 61.34%–70.21% of the TP_{FC} were identified. In contrast, only 33.69%–38.02% of TP_{FC} were identified in the PDY and DCY samples. The differences between TP_{FC} and TP_{HPLC} are due not only to the presence of unidentified polyphenolic antioxidants but also to the lack of specificity of the Folin-Ciocalteau assay since all substances with the catechol structure will give positive results.

3.2. Effect of industrial processing on the antioxidant activity of yerba mate extracts

Industrial processing modified ($P < 0.05$) the AI of the extracts (Fig. 2). Extracts from GLY and PDY samples had the highest AI values (75%) followed by the ZY and DCY extracts (66%). Forced aging had a detrimental effect since AI dropped from 66 to 45%. No consistent relationship was detected between the TP content and the AI values. GL and PDY leaves appear to be the most appropriate raw materials for extracting antioxidants, however, since the TP content of the GL extract is much lower (Table 1), the raw material requirements will be 22–25 times higher.

3.3. Antioxidant activity of the yerba mate extracts on bulk oil and oil/water emulsions

Fig. 3 shows the antioxidant effect of 2 doses of the yerba extracts (20 (Y20) and 60 μm/kg oil (Y60)), on the TBARS values of sunflower oil free of added antioxidants stored at 60 °C for 32 days. Twenty μmol/kg of a commercial extract rich in tocopherols (Toc20) was used as a positive control.

From day 4 to the end of the storage period, the Y20 and Toc20 treatments presented similar activities ($P < 0.05$). Increasing the

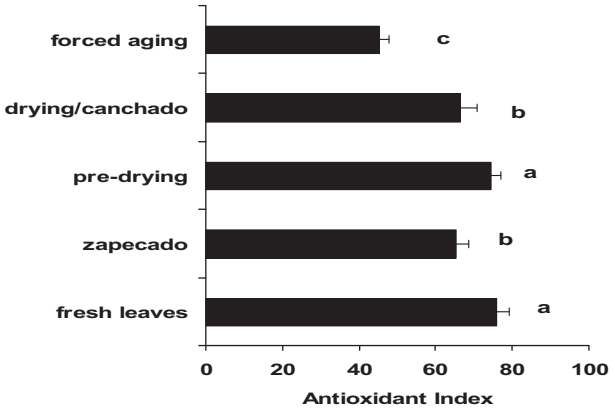

Fig. 2. Effect of industrial processing on the antioxidant index (AI) of the extracts. [a–c]Bars with the same letter are not significantly different ($P > 0.05$). Each point is the average of at least 3 replicates.

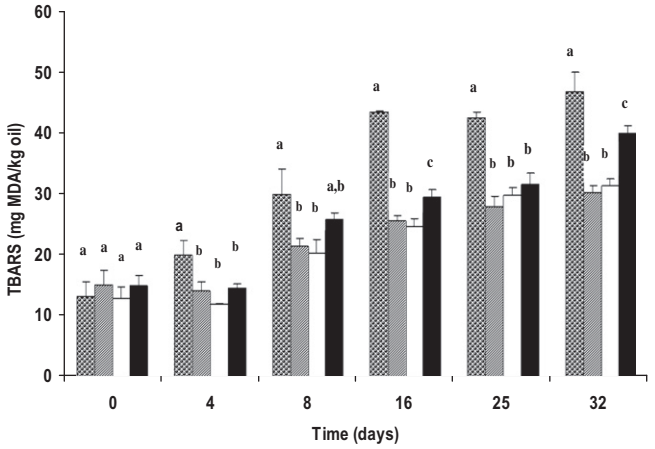

Fig. 3. Effect of 20 (Y20) and 60 (Y60) μmol/kg) of yerba and 20 μmol/kg (Toc20) tocopherol extracts on the TBARS formation of sunflower oil. Each point is the average of at least 3 replicates. [a–g]Means within each storage period having the same letter are not significantly different ($P > 0.05$). Control ▩; Toc20 ▨; Y20 □; Y60 ■.

dose of the yerba extract from 20 to 60 μmol/kg had negative effects since the efficiency of the extract was reduced by 27%.

In the case of the oil/water emulsions (Fig. 4), after 2 day storage no benefit in oxidative stability ($P > 0.05$) was detected with the Toc20 or the Y20 treatments; however, increasing the extract dose to 60 μmol/kg resulted in an oxidative stability growth of 35%.

3.4. Relationship between antioxidant activity and polyphenol composition

Because of the complexity of the experimental design, the relationship between polyphenol composition and antioxidant activity was focused only on the action of two caffeoyl derivatives (caffeic and chlorogenic) and three flavonoids (rutin, quercetin and kaempferol) commonly found in yerba mate (Filip, Lopez, Giberti, Coussio, & Ferraro, 2001).

3.4.1. Antioxidant activity of the individual polyphenols

The antioxidant activity of the individual polyphenols, (C, CL, K. Q, R) on β-carotene/linoleic acid stability showed that the most potent compound was quercetin, its AI value was 96.46%, followed by kaempferol and caffeic acid (83.45% each; Data not shown). No significant difference ($P > 0.05$) was detected between rutin and chlorogenic acid, their activity was 74.86% (Data not shown). Results also showed that in accordance with previous work done with radical quenching tests in model systems (Rice –Evans, Miller & Paganga, 1997; Shahidi & Wanasundara, 1992), the antioxidant activity of the polyphenols was strongly dependent on the number and position of free hydroxyl groups attached to the aromatic rings. Rice –Evans et al., (1997) concluded that the presence of the *o*-dihydroxyl (catechol) structure of the B-ring, the C2-C3 double bond in conjunction with a C4-oxo function and the C3 and C5-hydroxyl groups in quercetin C ring accounted for its extremely high antioxidant efficiency. Alterations of these arrangements and/or substitution of contributing hydroxyl groups by glycosylation or esterification will modify antioxidant activity.

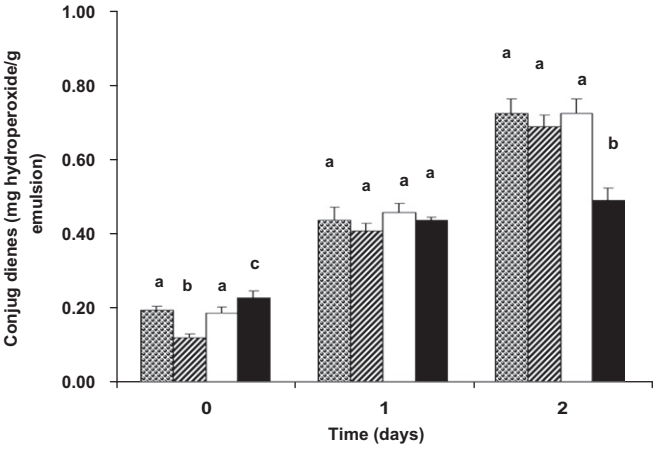

Fig. 4. Effect of 20 (Y20) and 60 (Y60) μmol/kg) of yerba and 20 μmol/kg (Toc20) tocopherol extracts on the conjugated dienes formation in oil/water emulsions. Each point is the average of at least 3 replicates. [a–c]Means within each storage period having the same letter are not significantly different ($P > 0.05$). Control ▩; Toc20 ▨; Y20 □; Y60 ■.

In accordance with Chaillou and Nazareno (2006), elimination of C3–OH in quercetin to form kaempferol diminished AI 13.8%. Glycosylation of the C3–OH in quercetin to produce rutin or esterification of the –CH = CH-COOH of caffeic to form chlorogenic acid reduced the activity by 21 and 10% respectively. Previous publications (Becker, Ntouma, & Skibsted, 2007; Fukumoto & Mazza, 2000) reported similar results using linoleic acid or methyl linoleate emulsions.

3.4.2. Mathematical model

Experimental data were satisfactorily modeled by Eq. (2); the coefficient of determination (R^2) was 0.997.

The final equation was:

$$\begin{aligned} AI_{pred} = {} & 75.24*xCL + 80.26*xC + 79.32*xR + 96.94*xQ \\ & + 84.26*xK - 50.99*xQxR - 63.43*xCxQ \\ & - 48.28*xCLxQ + 40.03*xCLxR + 35.07*xCxCL \\ & + 160.33*xKxQxR - 311.59*xCxKxR \\ & - 255.94*(xCLxQxR + xCxCLxK) \\ & - 206.74*(xCxCLxQ + xCxCLxR) \end{aligned} \quad (3)$$

Quercetin was the highest contributor to the linear term followed by kaempferol and caffeic acid while rutin and chlorogenic acid inputs were the lowest (79.32 and 75.24% respectively). We detected 11 significant interactions ($P < 0.05$), five of them were positive and six negative. The parameters of the CL*Q*R/C*CL*K and the C*CL*Q/C*CL*R pairs were similar ($P > 0.05$) therefore they were grouped and replaced by their respective average.

The validation tests done with different polyphenol blends (C/CL 50:50%; CL/Q 50:50%; CL/R 50:50%; CL/C/Q 25:25:50%) showed that the experimental and predicted values were in good agreement, the correlation coefficient was 0.99.

The main effects contribution to the antioxidant activity was much higher than the interactions components. In a five component system with equimolar polyphenol concentrations ($x_i = 0.20$), the predicted activity was 71.09%, the main effects accounted for 117% of the total activity, the interactions terms were negative and corresponded to 17% of AI_{pred}

To determine which were the best and worst performing antioxidant blends we calculated AI_{pred} of all the possible combination of two, three or four polyphenols assuming that within each combination, the individual components were at the same molar concentrations. Fig. 5 presents the total antioxidant index (AI_{pred}) as well as the main effects (AI1) and the sum of the 2nd (AI2) and 3rd (AI3) degree interaction terms of mixtures containing all the polyphenols (ALLP) and the best and worst performing systems.

No antagonistic effect was detected in the most efficient blends (K/Q, C/CL, CL/R, K/Q/R), in contrast, in the ALLP and the 4 less active mixtures (C/CL/Q/R, C/CL/Q, CL/Q/R, C/K/R) the negative

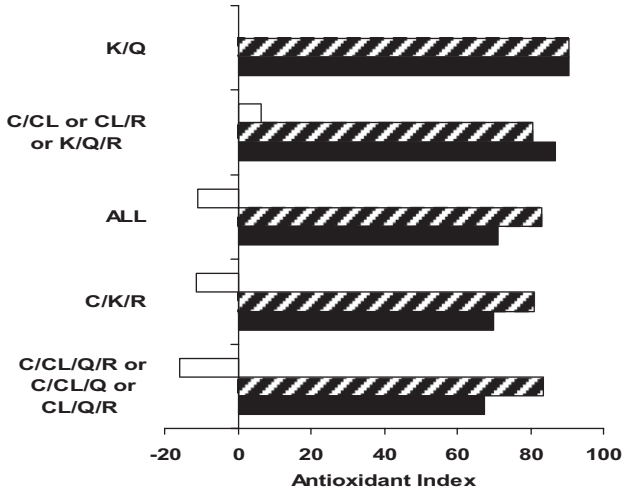

Fig. 5. Antioxidant index (AI) of the best and worst performing polyphenol combinations predicted by the model. Caffeic (C); Chlorogenic (CL); Quercetin (Q); Rutin (R) and Kaempferol (K). Within each treatment the relative concentrations of each polyphenol were equal.

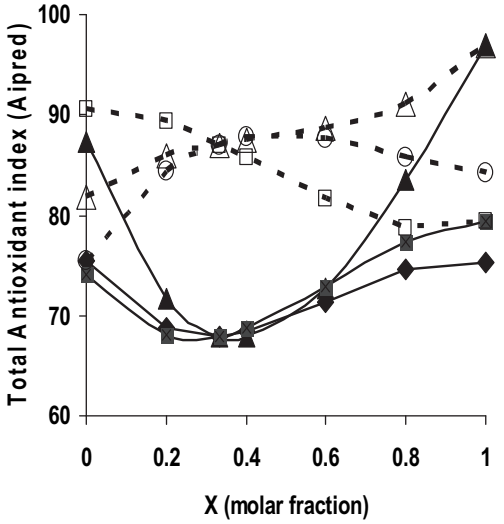

Fig. 6. Effect of the individual components molar fraction (x) on the predicted total antioxidant index (AI) of the kaempferol (K; ○)/quercetin (Q; ▲)/rutin (R; ■) and the chlorogenic (CL; ◆)/quercetin (Q; Δ)/rutin (R; □) systems.

interactions ranged from −10.83 to −15.97%. Differences in AI_{pred} between the ALLP and the least performing systems were very low (1.4–3%). Removal of K from the ALLP blend reduced AI_{pred} from 71.09 to 67.61%; further elimination of R, C or CL to form C/CL/Q, CL/Q/R or C/K/R/ did not cause substantial modifications in the total activity.

Taking away Q from the C/CL/Q or CL/Q/R systems improved AI_{pred} by 28% mainly through a considerable increment of the interaction contribution from an antagonistic value of −15.97 to a synergistic behavior of 6.35%. Replacing CL by K or eliminating C and CL from ALLP to form in both cases the K/Q/R system eliminated the antagonistic effect and consequently enhanced AI_{pred}. The best performing blend was K/Q with an AI_{pred} of 90.96%.

To analyze the variation of AI_{pred} with polyphenol concentration, we assumed that in the case of a three antioxidants mix, the molar fractions of one component varied from 0 to 1 (independent variable) and the contents of the other two were equal (dependent variables).

Fig. 6 shows the relationships between AI_{pred} and the relative contents of each polyphenol for the K/Q/R combination. This system has only two interactions, one positive, K*Q*R, and another negative, Q*R, therefore, variations in the total activity are caused mainly by changes in the main effects. Enriching the mix with 40%–60% of kaempferol, a poliphenol whose activity was marginally smaller than the AI_{pred} of the initial blend (K/R 50/50), improved AI_{pred} up to a maximum of 88%. This effect was due chiefly to an increment of the interaction component from −13 to 1.8% that overcompensated the marginal drop of the main effects (Data not shown). Further accretion resulted in a marginal loss in the predicted activity of 1%.

Increasing the level of Q, the most efficient antioxidant, improved the system's activity by 29%, mainly by an enhancement of the main effect that overcame the antagonistic behavior of the interactions detected for xQ values between 0.4 and 0.8 (See Fig. 7). In contrast, accumulation of rutin, the least active compound of the system, reduced the linear term and increased the antagonistic contribution of Q*R (Data not shown) resulting in an AI_{pred} loss of 12% (Fig. 6).

The efficiency of the Q/K combination improved linearly with Q accumulation from 84.25% to a maximum of 96.94% for pure Q (Data not shown).

Changes of AI_{pred} with polyphenols relative content in the C/CL and CL/R blends were similar (Data not shown); this behavior could be expected as the difference in antioxidant activity between both compounds, C and R, is very low. The best performing composition corresponded to a 50/50 mix.

Fig. 6 also shows the AI_{pred} dependence with polyphenol concentration for the CL/Q/R combination; the results obtained with the C/CL/R mix were similar (Data not shown). Increasing the molar fraction of Cl, Q or R to 0.3–0.4 reduced AI_{pred} to its minimum level of 68.41%. As in the K/Q/R system, Q incorporation had the strongest response in activity, AI_{pred} dropped from 87.3% to 68.41% followed by a rise to 96.46%. No significant differences in AI_{pred} were detected with Cl or C accumulation up to $x = 0.8$.

Fig. 8 shows the variations of the total activity (AI_{pred}) as well as the main (AI1) and total interactions (AI1 + AI2) effects with xQ in the CL/Q/R combination. Similarly to the K/Q/R mix (Fig. 7), AI1 increased with Q addition however, the response was almost 3

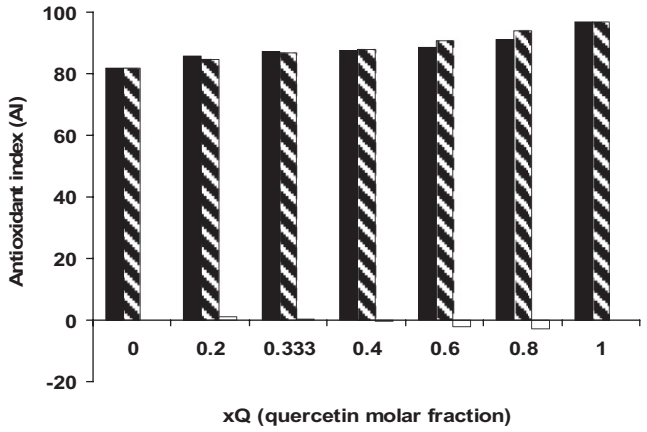

Fig. 7. Effect of quercetin molar fraction (xQ) on the total antioxidant activity (AIpred) and on the main (AI_1) and interaction effects (AI2 + AI3) of the kaempferol (K)/quercetin (Q)/rutin (R) system predicted by the model. AIpred ■; AI1 ▨; AI2 + AI3 □.

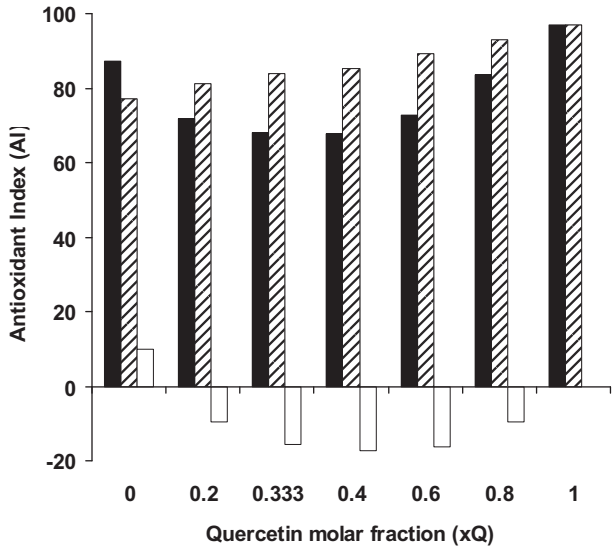

Fig. 8. Effect of quercetin molar fraction (xQ) on the total antioxidant activity (AIpred) and on the main (AI1) and interaction (AI2 + AI3) effects of the chlorogenic (CL)/quercetin (Q)/rutin (R) system predicted by the model. AIpred ■; AI1 ▧; AI2 + AI3 □.

times lower, 10.5% vs 29%, because of smaller differences in AI1 at xQ = 0 and at xQ = 1.

If we compare the total interactions contribution (AI1 + AI2) in both systems (Figs. 7 and 8), we can observe that the antagonistic effect is higher for the CL/Q/R combination, resulting in a reduction of the total activity that reached its minimum level (67.9%) at a CL/Q/R relative content of 40/30/30 (Fig.6).

CL or R accumulation reduced the main term input and enhanced the antagonistic effect up to a peak at xi = 0.2 followed by a gradual increase of the former and elimination of the latter (Data not shown). The combination of these two opposite effects explained the AI_{pred} variations observed in Fig. 7.

In the case of the C/K/R blend, the accumulation of any of the components had the same effect, AI_{pred} diminished until reaching a minimum level of 70% corresponding to a system composition of 20/40/40 or 40/30/30 (Results not shown).

4. Conclusions

Industrial processing modified the polyphenol content and composition as well as the capacity of the yerba mate extracts to inhibit the oxidation of the β-carotene/linoleic acid system.

The leaves from the pre-drying step were the most appropriate raw material because they combined maximum activity with high polyphenol content.

Twenty μmol/kg of the yerba mate extract inhibited MDA formation in sunflower oil, its efficiency was similar to the one obtained with a commercial extract rich in tocopherols. However, enhancing the dose to 60 μmol/kg resulted in an activity loss of 27.8%.

No effect on conjugated dienes formation was detected when 20 μmol/kg of the yerba or the tocopherol extracts were added to oil/water emulsions. On the other hand, increasing the dose of the yerba extract to 60 μmol/kg improved lipid stability.

The relationship between polyphenol composition and antioxidant activity of a mixture of caffeic, chlorogenic, kaempferol, quercetin and rutin was satisfactorily predicted with a polynomial model. Results showed that quercetin was the highest contributor to the linear term followed by kaempferol and caffeic acid while rutin and chlorogenic acid inputs were the lowest. The model detected five synergistic and six antagonistic effects.

The most effective combinations were K/Q, C/CL, CL/R and Q/K/R while C/CL/Q/R, C/CL/Q and CL/Q/R were the least efficient.

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