Research Article

Antioxidant activity of yerba mate extracts: Interactions between the individual polyphenols

Julia Valerga[1], Robin Shorthose[2] and Maria C. Lanari[1]

[1] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET – La Plata, Facultad de Ciencias Exactas Universidad Nacional de La Plata (UNLP), La Plata, Buenos Aires, Argentina

[2] CSIRO Food and Nutritional Sciences, Tingalpa DC, Qld, Australia

Yerba mate extracts contain strong antioxidants like chlorogenic (CL) and its derivatives, caffeic (C), quercetin (Q), rutin (R), and kaempferol (K) that may improve food products nutritional quality and rancidity. To obtain products with consistent activity and composition, we analyzed the effect of yerba's industrial processing on extracts composition, radical scavenging capacity (AA), inhibition of β-carotene/linoleic acid oxidation (AI), and ferric reducing/antioxidant power (RP). We also determined the relationship between the composition of a mixture of C, CL, K, Q, and R and their AA, RP, and AI values. Industrial processing modified polyphenol composition and antioxidant activity of the yerba extracts. Pre-dried and dried/canchada leaves were the most appropriate raw materials combining optimum AA, RP, and AI levels. Extract's capacity to improve ground beef's lipid stability was better than similar levels of α-tocopherol. Relationships between AA, RP, or AI and polyphenol composition were satisfactorily predicted by a quadratic, a full or a reduced cubic models, respectively. Simultaneous optimization of all models allowed determining the best and worst performing blends. Extracts contents of caffeic, chlorogenic and its derivatives, quercetin, and rutin were within or under the limits of the least active region predicted and may account for the low activity levels observed.

Practical applications: Due to its antioxidant and therapeutic properties; polyphenolic extracts from yerba mate leaves can improve the sensorial quality and shelf life of ground beef and sunflower oil as well as enhance the organism defense system. Commercial application of these extracts by the food and pharmaceutical industry requires products of consistent composition and activity. In this study we also determined the best raw material across the different processing steps and developed mathematical models that can be used to calculate the antioxidant activity of yerba extracts based on their polyphenol composition.

Keywords: Antioxidant / *Ilex paraguariensis* / Modelling / Polyphenols / Yerba mate

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Correspondence: Dr. Maria C. Lanari, Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Fac Cs Exactas UNLP, Calle 47 y 116 S/N°, La Plata B1900AJJ, Buenos Aires, Argentina
E-mail: cecilialanari@gmail.com
Fax: +54 221 425 4853

Abbreviations: AA, antiradical activity; **AH,** antioxidant; **AI,** antioxidant index; **AOA,** antioxidant activity; **C,** caffeic; **CL,** chlorogenic; **D,** desirability factors; **DCY,** "dried/canchada" yerba mate; **DL,** dried leaves; **DPPH***, 2,2-diphenyl-picryl-hydrazyl free radical; **FAY,** forced aged yerba mate; **FRAP,** ferric reducing antioxidant power; **GAE,** gallic acid equivalents; **GL,** yerba mate fresh green leaves; **hp,** hydroperoxide; **HPLC,** high performance liquid chromatography; **K,** kaempferol; **MDA,** malonedialdehyde; **PDY,** predried yerba mate; **Q,** quercetin; **R,** rutin; **RP,** reducing power; **TBARS,** 2-thiobarbituric acid reactive substances; **TOC,** tocopherol; **TP,** total polyphenol; **ZY,** zapecada yerba mate

1 Introduction

Several studies informed that yerba mate (*Ilex paraguariensis*) tea has antioxidant and hepatoprotective properties [1–3] as well as the capacity to improve the cardiovascular [4] and the central nervous systems [5]. Puangpraphant and Gonzalez de Mejia [5] also demonstrated that yerba mate phytochemicals inhibited pro-inflammatory markers. Some of these pharmacological properties 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) and to flavonoids like quercetin (Q), rutin (R), and kaempferol (K) [6].

Lipid oxidation is the main cause for the development of undesirable changes in flavor and appearance as well as the formation of toxic carcinogenic compounds in food products. In the last years there has been a considerable growth in 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. Recent reports showed that beside its positive health properties, polyphenolic extracts from yerba mate inhibited oxidative rancidity in salami [7], sunflower oil, and oil in water emulsion [8]. 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.

Appropriate selection of raw material and a thorough knowledge of the relationship between polyphenol composition and antioxidant activity (AOA) play a fundamental role in the obtention of extracts with consistent activity and composition, a key requisite for a successful commercial application of these products.

Industrial production of yerba mate involves a blanching step or ''zapecado'' followed by predrying 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% water content. Once dried the leaves 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. Recent reports showed that these processes modified the polyphenol content and composition of yerba mate leaves [8, 9] as well as their capacity to inhibit linoleic acid oxidation [8].

Due to the great variety and complexity of the antioxidants mechanism of action, Frankel and Meyer [10] recommended determining the antioxidant activity of natural extracts with several methods based on different mechanism of action. Prior et al. [11], reported that antioxidants can deactivate radicals by hydrogen and/or electron transfer and that linoleic acid peroxidation inhibition occurs mainly by quenching peroxyl radicals via hydrogen transfer.

Valerga et al. [8], reported that industrial treatments reduced yerba mate extracts capacity to inhibit linoleic acid peroxidation therefore to optimize raw material selection AOA dependence with industrial processing must be complemented with tests based on electron transfer mechanisms. The 2,2-diphenyl-picryl-hydrazyl free radical (DPPH*) [12] and the ferric reducing antioxidant power (FRAP) [13] assays fulfill this condition and have the advantages of being simple and easy to implement [10].

The relationship between the polyphenol composition of a blend C, CL, K, Q, and R and its capacity to inhibit linoleico acid peroxidation was satisfactorily predicted by a polynomial model calculated using statistical mixture design [8]. This method has the advantage of assessing potential synergistic/antagonistic effects and gives a better image of these interactions. Complementing this study with similar analysis using the DPPH* and FRAP tests will give a more thorough description of the polyphenol composition/AOA relationship.

The objectives of this study were:

(i) To run a comparative analysis of the effect of industrial processing on the AOA of yerba mate extracts determined with the DPPH*, FRAP, and β-carotene/linoleic acid oxidation assays to optimize raw material selection.
(ii) To determine the relationship between the compositions of a mixture of C, CL, K, Q, and R and its antioxidant activity using the three assays previously named.
(iii) To obtain the best and worst performing C, CL, K, Q, and R blends based on the DPPH*, FRAP, and β-carotene/linoleic acid oxidation results.

2 Materials and methods

2.1 Extracts

Samples from yerba mate fresh green leaves (GL) and from zapecada (ZY), predried (PDY), ''dried/canchada'' (DCY), and forced aged (FAY) yerba mate (*Ilex 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, DCY, and FAY samples were kept at 4°C.

After storage, the samples were ground to a fine powder in a coffee mill (Moulinex Corp., Buenos Aires) and extracted at least five times with 80% acetone/H_2O (w/v = 1/25) using a Soxhlet extractor [14]. The extracts were concentrated under reduced pressure with a rotary evaporator at 40°C. All determinations were made with freshly extracted samples.

2.2 Effect of industrial processing on the antioxidant activity of yerba mate extracts

The total polyphenol (TP) contents [mg of gallic acid equivalents (GAE)/g dried leaves (DL)] and the polyphenol composition of the extracts used in the current study, as well as their capacity to inhibit the β-carotene/linoleic acid oxidation (antioxidant index; AI) were reported previously [8].

The antiradical activity (AA) of the yerba mate extracts was measured with the DPPH* scavenging assay [12] with a reaction time of 80 min.

The reducing power (RP) was evaluated with the FRAP test described by Benzie and Strain [13] after 8 min of reaction time.

The concentration of antioxidant used in these assays was adjusted to have a TP final level of 25 μmol GAE/L. AA and RP were expressed as: (a) % DPPH/TP and (b) % Fe^{2+}/TP, respectively.

Since Valerga et al. [8], AI analysis was done using an extract level of 20 μmol GAE/L, to compare the AA, RP, and AI values, the antioxidant index was redetermined using 25 μmol GAE/L with Wettasinghe and Shahidi's protocol [15]. AI experimental results were also calculated as the percentage of the remaining β-carotene/(TP).

2.3 Antioxidant activity of the yerba extracts on ground beef

The beef samples were cut from the Longissimus muscle of five steers from a local abattoir. At 48 h post mortem, each muscle was cut into sections, vacuum-packaged and stored at −20°C in the dark until required for analysis (within 30 days). After removing the subcutaneous fat, sections of each muscle were ground with a mincer (Moulinex D-56; Buenos Aires, Argentina), using four cycles each of 5 s, and divided into 20 g patties.

Freshly obtained extracts from DCY samples were dissolved in 1 mL of methanol and added to each patty to a TP final concentration of 25 μmol GAE/kg meat. The control samples had 1 mL of methanol with no extract added. A similar level of a commercial extract with 50 g/100 g natural tocopherols (TOC) was used as a positive control.

Samples were wrapped in oxygen permeable PVC films (O_2 permeability = 15 500–16 200 cm^3/m^2/24 h at 23°C) and stored at 4°C for 0, or 6 days. At each storage period, lipid oxidation was determined using the 2-thiobarbituric reactive substances (TBARS) method [16] and expressed as mg malonedialdehyde (MDA)/kg meat.

2.4 Relationship between polyphenol composition and antioxidant activity

The relationships between AA or RP and polyphenol composition were determined using different combinations of caffeic (C), chlorogenic (CL), quercetin (Q), rutin (R), and kaempferol (K) (Sigma–Aldrich, Buenos Aires, ARG). The total antioxidant concentration (AH) of the blends was 25 μmol/L.

Valerga et al. [8], developed a model that predicted the relationship between the capacity to inhibit β-carotene/linoleic acid oxidation and the polyphenol composition using the same blend, and experimental design as in the current study but with a lower dose (AH = 20 μmol/L). As the equation is only valid within the experimental conditions applied for its obtention, to determine which will be the polyphenol systems which combine optimum levels of the three parameters (AA, AI, and RP), a new AI/polyphenol composition model was calculated using a total mixture content of 25 μmol/L.

2.5 Statistical analysis

Results are expressed as the mean and the standard deviation (SD) of at least three replications. The effect of industrial processing on the antioxidant activity (AA or RP) of the yerba mate extracts was analyzed using the SYSTAT software [17]. Significant differences among means were determined by one way ANOVA followed by pairwise comparisons with the Student's-t test; p-values ≤ 0.05 were considered significant.

The experimental design adopted to determine the relationship between the polyphenol composition and the antioxidant activity (AOA) consisted of a simplex lattice mixture design [18] replicated three times, with five factors (C, CL, K, Q, R) at four levels each. The design was generated by the Systat 12 software [17] and resulted in a total of 105 runs.

Antioxidant activity prediction was done using Scheffé's 3rd degree canonical model [18]:

$$\mathrm{AOA}_{\mathrm{pred}} = \sum_{i=1}^{n} \beta i\, xi + \sum\sum_{i<j}^{n} \beta ij\, xi\, xj + \sum\sum_{i<j}^{n} \delta ij\, xi\, xj\, (xi - xj) + \sum\sum\sum_{i<j<k}^{n} \beta ijk\, xi\, xj\, xk \quad (1)$$

where $\mathrm{AOA}_{\mathrm{pred}}$ represents the AA, AI, or RP values predicted by the model; βi and βijk are the coefficients for the linear and 3rd degree interaction terms, respectively. In this model, the 2nd degree interactions include quadratic (βij) and cubic (δij) coefficients. In the absence of the two ways interaction's cubic term, positive or negative values of the coefficients indicate either a synergistic or antagonistic effect, respectively [18].

The concentrations of the mixture components (i, j, k) were expressed as molar fractions (x). Estimation of the model parameters was done by automatic model regression using backwards elimination with the Design Expert 7 software (Stat-Ease, Minneapolis, MN, USA). To eliminate unnecessary terms from the model, within each group of effects (linear, 2nd or 3rd way interactions) nonsignificantly different coefficients ($p>0.05$) were grouped and replaced by their average.

Individual or multiple response optimizations were evaluated using the Desirability Function method with the Design Expert 7 Numerical Optimization Module.

$\mathrm{AA}_{\mathrm{pred}}$, $\mathrm{RP}_{\mathrm{pred}}$, and $\mathrm{AI}_{\mathrm{pred}}$ individual desirabilities (d_i) were determined with the equations proposed by Derringer

and Suich [19]. The desirability factor (d_i) corresponding to response (y_i) maximization was calculated as:

$$\begin{aligned} d_i &= 0 && y_i < \mathrm{Li} \\ d_i &= \left(\frac{y_i - \mathrm{Li}}{\mathrm{Ti} - \mathrm{Li}}\right)^s && \mathrm{Li} \le y_i \le \mathrm{Ti} \\ d_i &= 1 && y_i > \mathrm{Ti} \end{aligned} \quad (2)$$

On the other hand, the equation used to minimize y_i was:

$$\begin{aligned} d_i &= 1 && y_i < \mathrm{Ti} \\ d_i &= \left(\frac{y_i - \mathrm{Ui}}{\mathrm{Ti} - \mathrm{Ui}}\right)^s && \mathrm{Ti} \le y_i \le \mathrm{Ui} \\ d_i &= 1 && y_i > \mathrm{Ui} \end{aligned} \quad (3)$$

where y_i is the antioxidant activity predicted by the model, Li and Ui represent the acceptable minimal or maximal y_i and Ti is the target value depending on specified constrains. ''s'' and ''u'' are user defined factors that weigh the influence of the target value and the minimal or maximal limits.

To determine the best and worst performing blends with respect to: AA_{pred}, RP_{pred}, and AI_{pred}, we calculated the overall desirability factor (D; Eq. 4) as:

$$D = \left(d_1^{v_1}, d_2^{v_2}, \ldots, d_n^{v_n}\right)^{1/\sum_{i=1}^{n} v_2} = \left(\prod_{i=1}^{n} d_1^{v_1}\right)^{1/\sum_{i=1}^{n} v_1} \quad (4)$$

The desirability factors (D) were generated assigning all models a relative importance level (v_i) of 3.

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 antiradical activity or reducing power and the capacity to inhibit β carotene/linoleic acid peroxidation of yerba mate extracts

In a previous publication, Valerga et al. [8], analyzed the effect of industrial processing on the polyphenol content and composition of the samples used in the current study. The authors reported that zapecado was the only process that affected the total polyphenol level determined with the Folin-Ciocalteau method (TP_{FC}) [20]. TP_{FC} values increased from 4.15 mg GAE/g DL in the green leaves to 96.54 mg GAE/g DL in the ZY, PDY, DCY, and FAY samples [8].

HPLC analysis of the polyphenol profile (Table 1) [8] showed that CL and its derivatives (mono- and di-caffeoylquinic isomers; CLD) were the major components of the phenolic fractions with concentrations ranging from 28 to 63% of TP_{FC}. A similar behavior was observed in cooked sweet potatoes [21] and blanched artichokes [22]. Caffeic acid was detected in the GL, PDY, DCY, and FAY samples; on the other hand, R was present in the GL and ZY leaves while Q was identified only in the GL fraction (Table 1).

In accordance with Isolabella et al. [9], industrial processing caused a significant enhancement of CLD content from 1.59 to 62.49 mg/g DL. Ferracane et al. [22], showed that blanching, steaming, or frying enhanced artichokes chlorogenic acid derivatives concentration between 66 and 94%.

Table 2 presents the influence of industrial processing on the AA, RP, and AI levels of the yerba mate extracts. Overall, processing increased the antiradical activity and the reducing power of the extracts but reduced their capacity to inhibit the β carotene/linoleic acid peroxidation.

Although zapecado, predrying and drying/canchado steps enhanced the AA from 1.79 to 3.00 L μmol TP^{-1}, a significant loss of 26% was detected after storage.

Our results showed that predrying improved RP by 50% however; a small loss (16%) was detected after the drying/canchado stage that was overcompensated by forced aging since at this point RP reached its peak value.

Extracts from GLY and PDY samples had the highest AI values followed by the ZY and DCY extracts. Forced aging had a detrimental effect since AI dropped to its lowest level.

Ou et al. [23], reported that the FRAP method cannot detect the activity of compounds whose mechanism involves hydrogen donation. Foti et al. [24], suggested that

Table 1. Effect of the processing step on the composition (mg/g DL) of the yerba mate extracts [8]

Processing step	C	SD	CL	SD	R	SD	Q	SD	DCL	SD
GL	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
ZY	nd	nd	6.92^b	0.07	15.49^b	1.44	nd	nd	38.24^b	3.66
PDY	0.09^b	$8\ 10^{-3}$	14.5^c	1.32	nd	nd	nd	nd	23.43^c	2.44
DCY	0.16^b	1.33	13.01^c	1.47	nd	nd	nd	nd	20.51^c	2.13
FAY	7.3^c	0.71	1.13^d	0.12	nd	nd	nd	nd	62.49^d	6.45

GL, yerba mate fresh green leaves; ZY, yerba mate zapecada leaves; PDY, yerba mate predried leaves; DCY, yerba mate dried and canchada leaves; FAY, forced aged yerba mate leaves. C, caffeic; CL, chlorogenic; Q, quercetin; R, rutin; and DCL, mono-/di-caffeoylquinic isomers. SD, standard deviation; nd, not detected. Means within each column with different letters (a–d) differ significantly ($p < 0.05$).

Table 2. Effect of industrial processing on the antiradical activity (AAext), reducing power (RPext) and antioxidant index (AIext) of the yerba mate extracts

Proc. step	AAext (L μmol TP^{-1})	SD	RPext (L μmol TP^{-1})	SD	AIext (L μmol TP^{-1})	SD
GL	1.79[a]	0.09	2.43[a]	0.04	3.31[a]	0.24
ZY	2.13[b]	0.12	2.58[a]	0.26	2.50[b]	0.23
PDY	2.64[c]	0.04	3.75[b]	0.30	3.10[a]	0.18
DCY	2.97[c]	0.07	3.15[c]	0.15	2.77[b]	0.30
FAY	2.21[b]	0.02	4.85[d]	0.17	1.67[c]	0.16

GL, yerba mate fresh green leaves; ZY, yerba mate zapecada leaves PDY, yerba mate predried leaves; DCY, yerba mate dried and canchada leaves; FAY, forced aged yerba mate leaves; TP, total polyphenol content in gallic acid eq.; SD, standard deviation.
Means within each column with different letters (a–d) differ significantly ($p < 0.05$).

the $DPPH^*$/phenolic antioxidants reaction in strong hydrogen bond accepting solvents, such as ethanol or methanol, was mainly through an electron transfer process. On the other hand, antioxidants inhibit linoleic acid peroxidation by quenching peroxyl radicals via hydrogen transfer [10]. The difference in behavior observed between the AI and the AA/RP values suggests that many of the antioxidants that act by hydrogen transfer were destroyed during industrial processing. These observations also confirm Frankel and Meyer [10] recommendations regarding the need to test the antioxidant activity of natural extracts with several methods based on different mechanism of action.

No consistent relationship was detected between the activity values and the total polyphenol content indicating the presence of compounds that can react in the Folin-Ciocalteau assay but are inactive in the AA, RP, and AI determinations used in the current study.

Results indicated that leaves from the predrying step followed by those from the drying/canchado process appear to be the most convenient raw material for antioxidant production since they combine the optimum AA, RP, and AI levels.

3.2 Antioxidant activity of the yerba extracts on ground beef

Although the results from Section 3.1 indicated that the PDY samples combined the highest possible levels of AA, RP, and AI followed by the ones from the DCY leaves, not all yerba industrial plants include this stage hence this assay was done with the DCY extract.

Figure 1 shows the effects of 25 μmol/kg of the DCY and TOC extracts on the TBARS levels of ground beef stored for 6 days at 4°C. The yerba extract was the most efficient treatment with an inhibition capacity of 60%; in contrast TOC25 reduced the TBARS levels by 42%. Our results are in agreement with a previous research done in pork salami [7].

Previous studies [25, 26] demonstrated that caffeic and chlorogenic acids as well as quercetin and rutin improved lipid stability in minced fish and beef muscle. In a previous publication, Valerga et al. [8], showed that the DCY extracts had high contents of mono and caffeoyl quinic acid derivatives as well as a small level of caffeic acid. Therefore, we can conclude that the presence of C, CL, and its derivatives may explain yerba mate extracts capacity to improve lipid stability of ground beef.

3.3 Relationship between antioxidant activity and polyphenol composition

Because of the complexity of the experimental design, the relationship between AA, AI, or RP and the polyphenol composition 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 [27].

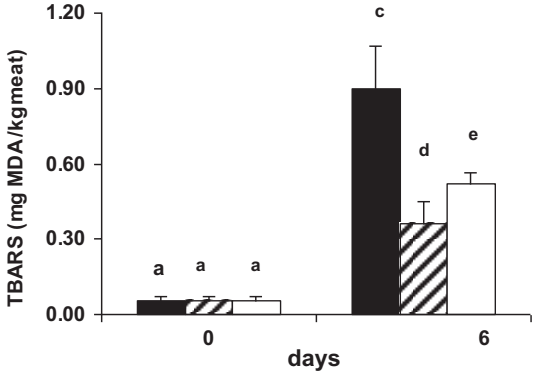

Figure 1. Effect of 25 μmol/kg of yerba (Y25) or tocopherol (Toc25) extracts on the TBARS formation of ground beef. (a–e) Bars within each storage period having the same letter are not significantly different (p>0.05). Each point is the average of at least three replicates. Control ■; Y25 ▨; Toc25 □.

3.3.1 Antioxidant activity of the individual polyphenols

Figure 2 shows the antiradical activity, reducing power, and antioxidant index of the individual polyphenols (C, CL, K, Q, and R). The rankings in decreasing efficiency were:

(i) Antiradical activity

$$Q > C \approx CL > R \approx K$$

(ii) Reducing power

$$Q > R > K > C \approx CLand$$

(iii) Antioxidant index

$$Q > C > CL \approx K \approx R$$

No significant differences ($p>0.05$) were detected between quercetin's AA, RP, and AI values which were between 5.01 and 4.58 μmol^{-1}. Rice-Evans et al. [28], concluded that quercetin's extremely high hydrogen or electron transfer capacity were due to the presence of certain structural elements like the *o*-dihydroxyl (catechol) structure of the B-ring, the C3 and C5-hydroxyl and the C2–C3 double bond combined with a C4-oxo function. Alterations of these arrangements and/or substitution of contributing hydroxyl groups by glycosilation or esterification will modify antioxidant activity [28].

Our results showed that glycosylation of quercetin's C3–OH to produce rutin reduced AI and RP 24, and 22% respectively; in contrast, the effect on antiradical activity was lower since AA diminished 10%. Previous reports also demonstrated that Q had higher DPPH* scavenging [29] and ferric reducing [30] activities as well as a better capacity for inhibiting β-carotene/linoleic acid peroxidation than R [31].

The removal of the C3'–OH from Q chemical structure to form kaempferol resulted in an RP drop of 30% whereas in the case of AA or AI, the reduction was 12–13%. Challiou and Nazareno [32] reached a similar conclusion using the β-carotene–linoleic acid system.

The esterification of caffeic's –CH=CH–COOH to form chlorogenic reduced only the AI levels by 10% without affecting AA or RP. Our results confirm previous publications reporting that caffeic and chlorogenic acids have similar DPPH* scavenging activity [33] and ferric reducing antioxidant power [34].

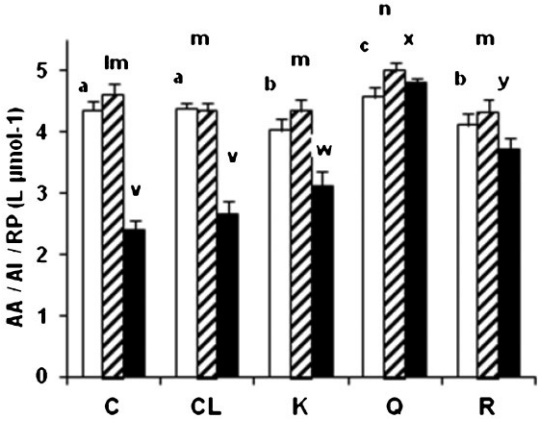

Figure 2. Antiradical activity (AA □), reducing power (RP ■) and antioxidant index (AI ▨) of 25 μmol/L of caffeic (C), chlorogenic (CL), quercetin (Q), rutin (R), and kaempferol (K). Within each assay type ($AA^{a–c}$, $AI^{l–n}$, $RP^{v–y}$) bars with the same letter are not significantly different ($p>0.05$). Each point is the average of at least three replicates.

3.3.2 Mathematical model

3.3.2.1 *Antiradical activity*

The 2nd degree cubic terms ($\delta ij\ xi\ xj(xi - xj)$) and the three ways interactions effects from Eq. (1) were not significant ($p>0.05$) therefore the model relating AA and polyphenol composition was reduced to a 2nd degree polynomial (Eq. 5) with a coefficient of determination (R^2) = 0.90.

$$\begin{aligned} AA_{pred} = {} & 4.16xC + 4.36(xCL + xQ) + 4.07xK + 4.23xR \\ & - 0.83(xC\,xCL + xC\,xR) + 0.73xC\,xQ \\ & + 0.94xCL\,xQ - 1.79xCL\,xR \end{aligned} \tag{5}$$

The validation tests done with different polyphenol blends ($xC/xCL = xCL/xQ = xCL/xR = 0.5{:}0.5$; $xCL/xC/xQ$ 0.25:0.25:0.50) showed that the experimental and predicted values were in good agreement, the correlation coefficient was 0.94.

The highest contributors to the linear term were Q and CL followed by R and C while kaempferol presented the lowest input. Although CL is one of the most potent compounds, its efficiency was lessened by its two negative interactions with R and C; in contrast, Q's individual input to AA_{pred} was improved by two synergistic effects: C × Q and CL × Q. The model also detected a third antagonistic interaction between caffeic acid and rutin.

The main effects inputs to the antiradical activity were much higher than the interactions contribution. In a five component system with equimolar polyphenols concentrations ($x_i = 0.20$), the predicted total activity was 4.16 L μmol AH^{-1}, the main effects accounted for 103% of the total activity while the interaction input was negative and corresponded to 3% of the AA_{pred}.

To determine the polyphenol composition of the best and worst performing blends we used the Design Expert 7 Numerical Optimization Module. Results showed that the

most active mixes did not contain R or K therefore, in these conditions the significant variables were caffeic, chlorogenic, and quercetin. The absence of rutin improved antiradical activity through an increment in the level of stronger antioxidants like Q or CL and the elimination of two negative interactions: C × R and C × R.

Figure 3 shows the response surface and the contour plots of AA_{pred} in the C/CL/Q system. Reduction of the caffeic level, the least active component, improved AA_{pred} by 10%, due to an enhancement of the relative proportions of the most potent antiradicals Q and CL and a diminution of the C × CL antagonistic effect. On the other hand, CL or Q concentrations higher than $x = 0.78$ reduced antiradical efficiency by 4.4%. The polyphenol compositions corresponding to the most efficient blends ($AA_{pred} \geq 4.50$ L μmol AH^{-1}) were: xC = 0–0.16 and xCL/xQ = 0.20–0.78.

The worst performing systems did not contain Q or K. Quercetin's exclusion affected AA_{pred} mainly through a considerable drop of the linear term combined with the elimination of the only two positive interactions CL × Q and C × Q. Figure 4 shows the influence of C/CL/R level on AA predicted by Eq. (5). The polyphenol content corresponding to the lowest activity area ($AA_{pred} \leq 3.84$ L μmol AH^{-1}) was: xC = 0–0.33; xCL = 0.27–0.56 and xR = 0.32–0.59.

Previous studies reported that CL's antiradical activity was equal or lower than that of the 1–3 dicaffeoylquinic acid [35] and that no significant differences were detected between the AA of the 4–5 and 3–5 caffeoylquinic acids [36]. Based on this information, we calculated the extracts AA_{pred} assuming that CL and DCL have similar linear coefficients (βi) and that the DCL interactions with the other components of the blend were marginal. The model accounted for 98–100% of the GL and FAY extracts' experimental AA. However, in the case of the ZY, PDY, DCY extracts, AA_{pred} was between 60 and 80% of their corresponding experimental values. These disparities could be due to the presence of unidentified antioxidants formed during industrial processing that were detected by the HPLC [8].

3.3.2.2 Reducing power

The relationship between RP and polyphenol composition of the C/CL/K/Q/R system was satisfactorily modeled by Eq. (1); the coefficient of determination (R^2) was 0.901.The final equation was:

$$\begin{aligned} RP_{pred} = {} & 2.34xC + 2.51xCL + 3.05xK + 4.96xQ + 3.81xR \\ & + 5.98xCL\,xQ + 1.97xCL\,xK + 6.21xC\,xQ \\ & + 2.36xC\,xK + 2.80xQ\,xR + 4.68(xK\,xR + xK\,xQ) \\ & + 7.22(xC\,xCL\,xR + xCL\,xQ\,xR) + 10.28xC\,xCL\,xQ \\ & + 15.11xC\,xCL\,xK + 11.87xCL\,xK\,xR \\ & + 8.18xC\,xQ\,xR - 8.02xK\,xQ\,xR \\ & + 5.99(xCL\,xQ(xCL - xQ) + xC\,xQ(xC - xQ)) \\ & - 6.38xCL\,xK(xCL - xK) + 2.68xR\,xQ(xR - xQ) \\ & - 4.07xQ\,xK(xQ - xK) \end{aligned} \quad (6)$$

The validation tests, done with polyphenol blends similar to the ones used for the AA_{pred} model, showed that the experimental and predicted values were in good agreement, the correlation coefficient was 0.95.

The linear coefficients (βi) indicated that the flavonoids (K, Q, and R) were stronger reducing agents than the caffeoyl derivatives (C and CL). Quercetin was by far the most effective compound followed by rutin and kaempferol, while caffeic and chlorogenic acids were the least active. The K × R and C × K effects were synergistic; however,

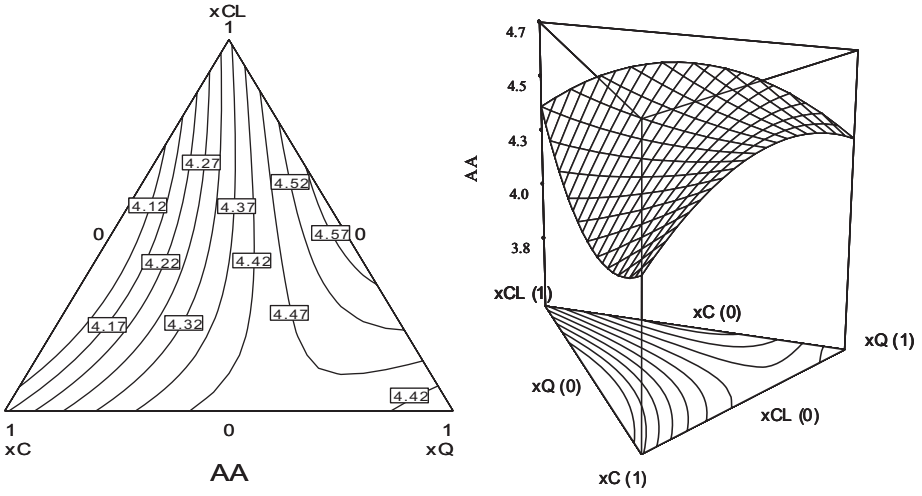

Figure 3. Surface plots and contour lines showing the dependence of the antiradical activity predicted by the quadratic model (AA_{pred}; L μmol AH^{-1}) on the caffeic (xC), chlorogenic (xCL), and quercetin (xQ) molar fractions.

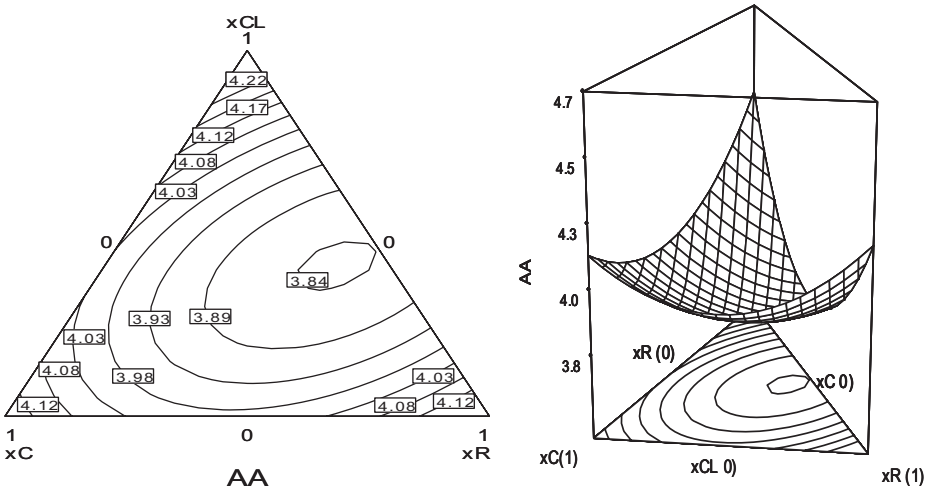

Figure 4. Surface plots and contour lines showing the dependence of the antiradical activity predicted by the quadratic model (AA_{pred}; L μmol AH^{-1}) on the caffeic (*x*C), chlorogenic (*x*CL), and rutin (*x*R) molar fractions.

the behavior (synergistic or antagonistic) of the CL × Q, CL × K, C × Q, Q × R, and Q × K interactions cannot be deducted directly from the βij and δij values since it also depends on the relative concentrations of the interactions components. We detected six synergistic and one antagonistic (K × Q × R) ternary effects.

Comparison of the AA and RP models (Eqs. 5 and 6) showed that the number of significant interactions was much higher in the RP than in the AA determination. Both methods are based on electron transfer mechanisms [37] however, the FRAP test is less selective since every compound with a redox potential ≤0.70 V will give positive results [13] and as a result may increase the complexity of the model.

Assuming an equimolar C/CL/K/Q/R mix ($x_i = 0.20$), we observed that the interactions input was much higher than for the AA_{pred} case since the RP_{pred}'s two and three ways effects were synergistic and accounted for 23 and 9% of the total activity, respectively.

The composition of the best and worst performing mixes was determined as described in the Section 3.3.2.1. Results showed that the most efficient systems had an *x*Q mean level = 0.43 ± 0.02 and none of them contained K suggesting that high Q concentrations were an important requirement to maximize reducing power. Figure 5 shows the response surface and the contour plot describing the RP_{pred} dependence with the C/CL/Q contents considering a constant value of *x*R = 0.15. Increasing *x*Q level from 0 to 0.40–0.50 resulted in a sharp improvement of the reducing power from a range of 2.63–2.82 to 5.16 L μmol AH^{-1}; further increments from 0.50 to 0.85 caused a small decline of 4.8%.

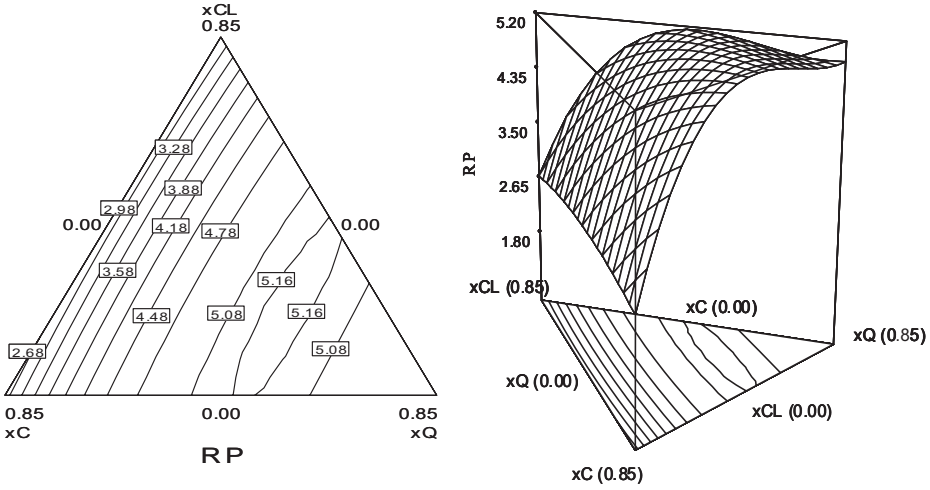

Figure 5. Surface plots and contour lines showing the dependence of the reducing power predicted by the full cubic model (RP_{pred}; L μmol AH^{-1}) on the caffeic (*x*C), chlorogenic (*x*CL) and quercetin (*x*Q) molar fractions. xRutin constant = 0.15.

The effect of C or CL enrichment depended on the amount of Q present in the system. For xQ contents higher than 0.30, changes in C and/or CL concentrations produced marginal variations $< 3\%$. On the other hand, in systems with low Q, modifying the C/CL levels altered RP_{pred} by almost 90% (data not shown). The C/CL/Q/R concentrations corresponding to $RP_{pred} \geq 5.16$ L μmol AH^{-1} were $x\mathrm{C} = 0$–0.40, $x\mathrm{CL} = 0$–0.42 and $x\mathrm{Q} = 0.40$–0.50 and $x\mathrm{R} = 0.15$.

Systems containing $x\mathrm{CL} = 0.82$–0.93 and $x\mathrm{K} = 0.07$–0.18 were the least active blends with $RP_{pred} = 2.27$–2.31 L μmol AH^{-1}; the main effects accounted for 114% of the total activity while the CL × K effect was antagonistic and corresponded to 14% of the RP_{pred}.

The reducing power of the C/CL system was also low (2.34–2.51 μmol AH^{-1}), rutin incorporation resulted in a continuous RP_{pred} enhancement up to 3.81 L μmol AH^{-1} (data not shown).

3.3.2.3 Antioxidant index

The cubic components of the two ways interactions terms in Eq. (1) were not significant; therefore, the antioxidant index (AI_{pred}) of the polyphenol blend was satisfactorily predicted by a reduced cubic model (Eq. 7):

$$\begin{aligned} AI_{pred} = {} & 4.56x\mathrm{C} + 4.47x\mathrm{CL} + 4.45x\mathrm{K} + 4.93x\mathrm{Q} + 4.31x\mathrm{R} \\ & - 1.29x\mathrm{C}\,x\mathrm{CL} - 1.67(x\mathrm{C}\,x\mathrm{K} + x\mathrm{CL}\,x\mathrm{K}) \\ & - 4.37x\mathrm{C}\,x\mathrm{Q} - 1.11x\mathrm{C}\,x\mathrm{R} - 4.27x\mathrm{CL}\,x\mathrm{Q} \\ & - 1.08x\mathrm{K}\,x\mathrm{Q} - 0.88x\mathrm{K}\,x\mathrm{R} - -3.46x\mathrm{Q}\,x\mathrm{R} \\ & - 14.73x\mathrm{C}\,x\mathrm{K}\,x\mathrm{R} - 7.64x\mathrm{C}\,x\mathrm{CL}\,x\mathrm{K} - 4.36x\mathrm{C}\,x\mathrm{CL}\,x\mathrm{Q} + \\ & + 6.17x\mathrm{CL}\,x\mathrm{K}\,x\mathrm{Q} - 9.91x\mathrm{CL}\,x\mathrm{K}\,x\mathrm{R} - 8.41x\mathrm{CL}\,x\mathrm{Q}\,x\mathrm{R} \\ & + 14.04x\mathrm{K}\,x\mathrm{Q}\,x\mathrm{R} \end{aligned} \quad (7)$$

The validation tests done as described in Section 3.3.2.1 showed that the experimental and predicted values were in good agreement with a correlation coefficient of 0.95.

Quercetin was the most effective inhibitor of linoleic acid oxidation followed by caffeic, chlorogenic, and kaempferol while rutin was the least active. The model detected 14 antagonistic interactions and only two synergistic effects, CL/Q/K and K/Q/R. When we compared Eq. (7) with the model previously reported by Valerga et al. [8], we noticed that in the current study the number of significant interactions was higher. These disparities could be caused by variations in the total antioxidant content utilized since the amount we used was 25% higher and may increase the significance of certain effects.

Assuming an equimolar C/CL/K/Q/R mix ($x_i = 0.20$), the AI_{pred} was 3.55 L μmol AH^{-1}, the main effects accounted for 127.8% of the total activity and the interactions input was antagonistic and corresponded to 27.8% of the total AI_{pred}.

Eq. (4) optimization showed that the majority of the best performing blends ($AI_{pred} = 4.50$–4.92 L μmol AH^{-1}) did not contained caffeic or chlorogenic hence, the relevant variables could be reduced to kaempferol, quercetin, and rutin. Caffeic acid levels higher than $x = 0.97$ combined with chlorogenic or rutin also reached AI_{pred} values of 4.50 L μmol AH^{-1}. These results are in accordance with Valerga et al. [8], who reported that the C/CL, C/R, and the K/Q/R blends were among the most effective combinations inhibitors of the β carotene/linoleic acid oxidation. Although C and CL are individually more effective than K or R, they are also part of 11 antagonistic effects and as a result, their elimination increased the activity of the system.

Figure 6 shows the influence of the K/Q/R relative concentrations on the response surface and the contour plots of the AI_{pred} considering $x\mathrm{C} = x\mathrm{CL} = 0$. In accordance

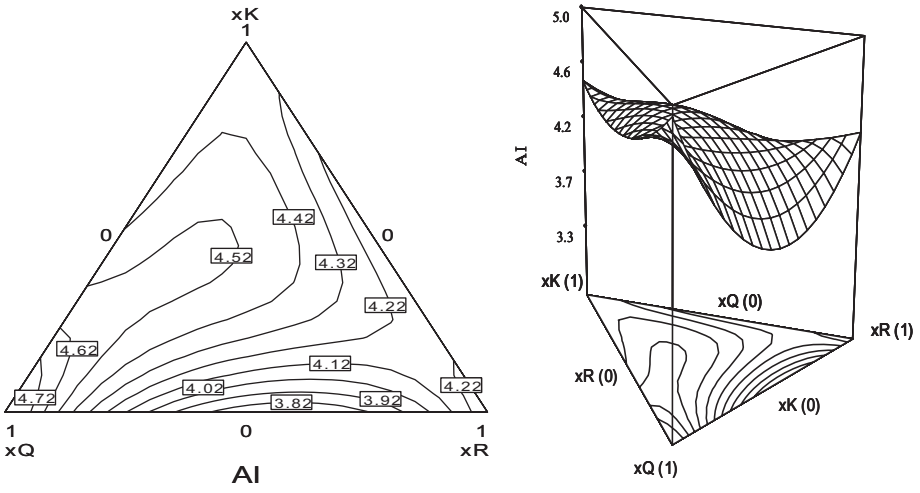

Figure 6. Surface plots and contour lines showing the dependence of the antioxidant index predicted by the reduced cubic model (AI_{pred}; L μmol AH^{-1}) on the kaempferol (xK), quercetin (xQ) and rutin (xR) molar fractions.

with Valerga et al. [8], the most active compositions were xK(0–0.22)/xQ(0.77–1)/xR(0–0.08). Enhancing the xK content from 0 to 0.21 to Q/R combinations containing xQ = 0.25–0.55 caused a sharp activity improvement from 3.73 to 4.42 L μmol AH^{-1}; further increments up to $x = 0.32$ only caused a 2.2% gain. In contrast, Q or R incorporation resulted in an activity increase ≤11% or a drop of similar magnitude, respectively.

The least efficient polyphenol combinations (AI = 3.35–3.38 L μmol AH^{-1}) contained only caffeic, chlorogenic, quercetina, and rutin. These results confirm Valerga et al. [8], study who reported that the C/CL/Q/R, C/CL/Q, and CL/Q/R mixes were the least effective combinations of the C/CL/K/Q/R system. Eq. (7) indicated that since K was part of all the existing synergisms, its absence may account for these results.

Q incorporation to a C/CL blend produced a sharp drop in AI_{pred} from 4.22–4.28 L μmol AH^{-1} to a minimum of 3.39 L μmol AH^{-1} which corresponded to a polyphenol composition of xC = 0.38–0.41; xCL = 0.23–0.39x and xQ = 0.34–0.45 (Fig. 7). In these conditions, the interactions negative contribution accounted for 38% of the total activity. Further Q addition improved the activity to a maximum of 4.93 L μmol AH^{-1} (pure Q) mainly through an enhancement of the main effects (Fig. 7).

When we analyzed the relationship between the antioxidant index and the composition of the C/CL/R system we observed that replacing Q by R reduced AI_{pred} dependence with the polyphenol composition as the predicted activity varied from 4.14 to 4.56 L μmol AH^{-1} (data not shown). Although Q per se was the strongest inhibitor of linoleic oxidation, its effect was significantly diminished by its negative interactions with C and/or CL. In the C/CL/R system, the antagonistic interactions at the lowest Ai_{pred} values were 7% of the total activity compared to 38% in the C/CL/Q blend.

3.3.2.4 Optimization of the polyphenol composition

Simultaneous optimization of Eqs. (5)–(7) allowed us to determine the best and worst performing blends with respect to the three parameters: AA, RP, and AI.

The solutions of the optimization process indicated that the most active systems were composed of (a) K, Q and/or R or (b) C, CL, and/or Q. In both systems the highest levels of desirability ($D = 0.87$) corresponded to xQ = 1. Considering $D = 0.71$ as the limit of acceptability, the K/Q/R compositions and predicted activity levels that fulfil this condition were:

$$x\mathrm{K}(0-0.49)/x\mathrm{Q}(0.32-1)/x\mathrm{R}(0-0.29);$$
$$\mathrm{AA_{pred}} = 4.36 - 4.61\,\mathrm{L\,\mu mol\,AH^{-1}},$$
$$\mathrm{RP_{pred}} = 4.96 - 5.58\,\mathrm{L\,\mu mol\,AH^{-1}} \text{ and}$$
$$\mathrm{AI_{pred}} = 4.93 - 5.03\,\mathrm{L\,\mu mol\,AH^{-1}}$$

In the case of the C/CL/Q system, the concentration range corresponding to $D \geq 0.72$ was much smaller than in the K/Q/R mix, the polyphenol contents were:

$$x\mathrm{C}(0-0.12)/x\mathrm{CL}(0-0.12)/x\mathrm{Q}(0.88-1).$$
$$\mathrm{AA_{pred}} = 4.42\ \mathrm{L\ \mu mol\ AH^{-1}},$$
$$\mathrm{RP_{pred}} = 4.72\ \mathrm{L\ \mu mol\ AH^{-1}} \text{ and}$$
$$\mathrm{AI_{pred}} = 4.42\ \mathrm{L\ \mu mol\ AH^{-1}}.$$

The polyphenol composition and antioxidant activities of the least active blends were:

$$x\mathrm{C}(0.17-0.50)/x\mathrm{CL}(0.27-0.50)/x\mathrm{R}(0.11-0.43);$$
$$\mathrm{AA_{pred}} = 3.85 - 3.99\,\mathrm{L\,\mu mol\,AH^{-1}},$$
$$\mathrm{RP_{pred}} = 2.73 - 3.16\,\mathrm{L\,\mu mol\,AH^{-1}} \text{ and}$$
$$\mathrm{AI_{pred}} = 4.07 - 4.39\,\mathrm{L\,\mu mol\,AH^{-1}}.$$

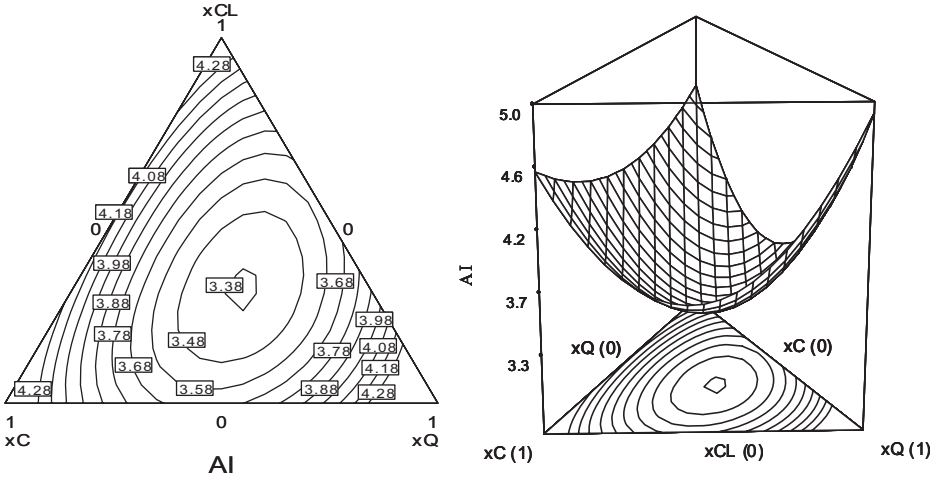

Figure 7. Surface plots and contour lines showing the dependence of the antioxidant index predicted by the cubic model (AI_{pred}; L μmol AH^{-1}) on the caffeic (xC), chlorogenic (xCL) and quercetina (xQ) molar fractions.

Table 3. Antiradical activity (AA_{pred}), reducing power (RP_{pred}), antioxidant index (AI_{pred}), and composition of the best and worst performing blends predicted by the models

Composition (molar frac)	AA_{pred} (L μmol AH^{-1})	RP_{pred} (L μmol AH^{-1})	AI_{pred} (L μmol AH^{-1})
*x*C[0–0.16]/*x*[CL + DCL] [0.18–0.80]/*x*Q [0.20–0.50]	4.42	4.72	4.42
*x*K[0–0.17]/*x*Q [0.63–1]/*x*R[0–0.29]	4.35–4.61	4.96–5.58	4.93–5.03
*x*C[0.17–0.33]/*x*[CL + DCL] [0.27–0.50]/*x*R[0.32–0.43]	3.85–3.99	2.73–3.16	4.07–4.39

AH, antioxidant; C, caffeic; CL, chlorogenic; Q, quercetin; R, rutin; DCL, mono-/di-caffeoylquinic isomers.

Results from Section 3.3.2.1 showed that Eq. (5) accounted for 98–100% of the GL and FAY extracts' experimental AA and for 60–80% of the ZY, PDY, and DCY extracts. In contrast, due to the lack of information regarding CLD's reducing power and antioxidant index, the predictability of Eq. (6 and 7) were much lower, ranging from 11 to 65%. Table 1 showed that CLD were the most abundant antioxidants in the yerba extracts, therefore, the solutions obtained through global optimization cannot be applied directly in our experimental conditions. To determine the compositions ranges of the best and worst performing mixes, the global optimization solutions obtained in the current section were compared with those corresponding to AA_{pred} (Section 3.3.2.1) and the polyphenol compositions were determined as the cross section between the results of the global and AA optimizations; the results obtained following these procedures are shown in Table 3.

Comparison of the AA, RP, and AI ranges predicted for the best and worst performing blends with those from the GL, ZY, PDY, DCY, and FAY extracts (Table 2) showed that the extracts AA and AI values and the RP values of the GL and ZY fractions were lower than the solutions predicted for the least active systems. This behavior can be explained considering that the CL and DCL total content in all fractions and the R concentration in the GL extract (Table 1) were within the limits of the least active region predicted by the models optimization. The GL fraction also contained an extremely active compound like Q (Table 1); however, its content was not high enough to make a significant impact. These two factors combined with C and R's extremely low levels can explain the low activity levels of the yerba extracts.

On the other hand, the RPs of the DCY samples and those from the PDY and FAY were higher than those from the GL and ZY fractions (Table 2) and fell within the range of the least and most efficient mixes, respectively. This improvement in performance may be due to the presence of compounds with a redox potential $\leq$0.70 V that can increase RP without influencing AA or AI.

4 Conclusions

Industrial processing improved the antiradical activity and the reducing power of the yerba mate extracts. In contrast, their capacity to inhibit linoleic acid peroxidation was significantly reduced. AA and RP assays involve electron transfer antioxidant while inhibition of linoleic acid peroxidation requires compounds that act via hydrogen transfer. The difference in behavior observed between the AI and the AA/RP values suggests that many of the antioxidants that act by hydrogen transfer were destroyed during industrial processing.

The leaves from the predrying and the drying/canchado stages appear to be a viable source of antioxidants since they combine the optimum AA, RP, and AI levels.

The capacity of the yerba extract to improve lipid stability in ground beef was better than a similar dose of α-tocopherol.

The relationships between polyphenol composition of a mixture of C, CL, K, Q, and R and their AA, RP, or AI values were satisfactorily predicted by a 2nd degree equation, a full or a reduced cubic models, respectively.

Assuming that CL and DCL individually have similar AA values and that the DCL interactions with the other components of the blend were marginal, the AA/composition model accounted for 60–100% of the GL, ZY, PDY DCY, and FAY extracts experimental AA.

Desirability analysis of the three models allowed us to determine the best and worst performing blends with respect to AA, RP, and AI.The solutions obtained for the high activity systems were:

(a) $x\mathrm{C}(0-0.16)/x(\mathrm{CL}+\mathrm{DCL})(0.18-0.80)/x\mathrm{Q}(0.20-0.50)$; $\mathrm{AA_{pred}} = 4.42\,\mathrm{L\,\mu mol\,AH^{-1}}$, $\mathrm{RP_{pred}} = 4.72\,\mathrm{L\,\mu mol\,AH^{-1}}$ and $\mathrm{AI_{pred}} = 4.42\,\mathrm{L\,\mu mol\,AH^{-1}}$

(b) $x\mathrm{K}(0-0.17)/x\mathrm{Q}(0.63-1)/x\mathrm{R}(0-0.29)$; $\mathrm{AA_{pred}} = 4.35-4.61\,\mathrm{L\,\mu mol\,AH^{-1}}$, $\mathrm{RP_{pred}} = 4.96-5.58\,\mathrm{L\,\mu mol\,AH^{-1}}$, $\mathrm{AI_{pred}} = 4.93-5.03\,\mathrm{L\,\mu mol\,AH^{-1}}$.

The polyphenol contents of the least efficient blends were:

$x\mathrm{C}(0.17-0.33)/x(\mathrm{CL}+\mathrm{DCL})(0.27-0.50)/x\mathrm{R}(0.32-0.43)$;
$\mathrm{AA_{pred}} = 3.85-3.99\,\mathrm{L\,\mu mol\,AH^{-1}}$,
$\mathrm{RP_{pred}} = 2.73-3.16\,\mathrm{L\,\mu mol\,AH^{-1}}$ and
$\mathrm{AI_{pred}} = 4.07-4.39\,\mathrm{L\,\mu mol\,AH^{-1}}$.

The C, CL, DCL, Q, and R contents of the extracts were within the limits of the least active region predicted by the models optimization or lower and may account for the extracts low activity levels.

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