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Multi-response optimization of phenolic antioxidants from white tea (*Camellia sinensis* L. Kuntze) and their identification by LC–DAD–Q-TOF–MS/MS

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ABSTRACT

The aim of this study was to model the extraction of phenolic antioxidants and identify the principal individual phenolic compounds of white tea by LC-DAD-MS/MS. A Box-Behnken design was applied to evaluate the effects of time, temperature and ethanol concentration in the extraction of phenolics and *in vitro* antioxidant activity. All mathematical models proposed by multiple regression analysis were significant ($P < 0.001$) and could explain up to more than 85% of the variance ($R^2_{adj} > 0.80$). A simultaneous optimization was performed using DPPH, ABTS, FRAP, (−)-epigallocatechin gallate, and (−)-epicatechin gallate to maximize the phenolic extraction and suggested optimum conditions of 10 min, 66 °C and 30% ethanol solution, with absolute error lower than 7%. The suggested optimum conditions were confirmed by external validation. The principal individual compounds identified by mass spectrometry were gallic acid, 5-galloylquinic acid, caffeine, theobromine, gallocatechin, epigallocatechin, epicatechin, epigallocatechin gallate, and epicatechin gallate.

1. Introduction

Considered the second beverage most popular after water, tea has received continual interest due to many beneficial health effects that include reducing the risks of cancer, type 2 diabetes and cardiovascular diseases (Kris-Etherton & Keen, 2002; Mukhtar & Ahmad, 2000; Pinto, 2013), besides the antimicrobial, anti-inflammatory and antioxidant capacities that are intensively related (Bansal et al., 2013; Zielinski et al., 2014). A large number of diversified types of teas are produced around the world among which the most popular are black, green, red, yellow, and white teas. The difference between white tea and others, is that the production of white tea (unfermented) is performed using new growth buds and young leaves which are harvested and dried immediately after (Hilal & Engelhardt, 2007).

The flavan-3-ols are principal bioactive compounds found in unfermented teas from *Camellia sinensis* that exhibit antioxidant activity and free radical scavenging activity (Aron & Kennedy, 2008). Among these, the principal compounds are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epicatechin (EC) and (+)-catechin (Wang, Provan, & Helliwell, 2000). In recent studies published by Unachukwu, Ahmed, Kavalier, Lyles, and Kennelly (2010), and Zhao et al. (2011), white tea showed high content of functional compounds and antioxidant activity compared, for example, to the green tea. Therefore, white tea can be a good resource for supplying bioactive compounds with functional properties.

In past years, the link between health problems and preservatives in foods has led to a decrease in the levels permitted with vegetable extracts becoming a new source for the food industry replace the synthetic antioxidants and to supplement the products with bioactive compounds. It is common to find different methods

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of extraction being used to extract the phenolic compounds from plant materials. Among the most common extraction method that have been mentioned in the literature is the use of solvents such as ethanol, acetone or methanol, or a mixture with water (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Guitiérrez, 2010). However, for food application, phenolics are preferably extracted with ethanol because it is regarded as food grade, biocompatible and more economical than other solvents (Ilayaraja, Likhith, Babu, & Khanum, 2015). During the extraction process some variables need to be evaluated including temperature, time and solvent concentration; therefore an optimization of the process is essential in order to reach the maximum potential of extraction. Response surface methodology (RSM) coupled with multiple linear regression is one of the tools used for optimization as it is able to evaluate multiple parameters and their interactions and to obtain mathematical models that are used to define the relationships between the response and the independents factors (Bas & Boyaci, 2002).

Therefore, the aims of this study were i) to model using multiple linear regression coupled with response surface methodology the extraction of phenolic antioxidants from white tea, and ii) to identify the individual phenolic compounds in the extract optimized using LC–DAD–Q-TOF–MS/MS.

2. Material and methods

2.1. Materials

White tea sample was kindly donated by Herbaflora (São Paulo, Brazil). According to the manufacturer, it was imported from China. A certificate of botanical authenticity of these leaves can be obtained directly from the producers. Phenol reagent, Trolox (6-hydroxy-2,5,7,8-tetremethychroman-2-carboxylic acid), TPTZ (2,4,6-tri (2-pyridyl)-s-triazine), DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), AAPH (2,2′-azobis(2-amidinopropane) hydrochloride), fluorescein, and chemical HPLC-grade standards (purity $\geq$ 95%) of gallic acid, theobromine, caffeine, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, (−)-epicatechin gallate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol, ethanol, acetic acid and acetonitrile were purchased from Fischer Scientific (Whitby, Ontario, Canada). Other reagents used in the experiments were all of analytical grade.

2.2. Methods

2.2.1. Extraction of phenolic antioxidants

Briefly, 100 g of dried white tea was mixed and the sample were ground using a pestle and mortar to obtain a homogenous fine powder that passed through 0.5 mm screen. In order to optimize the conditions for the extraction of phenolic compounds from the white tea, 1 g of the powdered tea was extracted with 50 mL of ethanol solution following a Box-Behnken design with 15 experiments. The factors (independent variables) studied for the extraction of phenolic antioxidants were: time (min, X_1), temperature (min, X_2), and ethanol concentration (%, X_3), at three levels of variation (Table 1). The sequence of experiments was performed randomly, and the samples were centrifuged (8160 $\times$ *g*, 20 min at 4 °C) (Sorvall RC-6-Plus, Fisher Scientific, Asheville, NC, USA) following extraction and transferred to Falcon tubes and immediately frozen at −20 °C until further analysis.

2.2.2. Determination of total phenolic compounds (TPC)

Total phenolic content was determined in the extracts according to the Folin-Ciocalteu procedure described by Singleton and Rossi (1965) with modifications. For this purpose, the sample absorbance values were compared against a calibration curve of gallic acid (GA) and the results were expressed as mg of gallic acid equivalents (GAE) per gram of tea [mg GAE/g].

2.2.3. Determination of total flavonoids content (TFC)

Total flavonoid content was quantified using an aluminum chloride colorimetric assay (Jia, Tang, & Wu, 1999). Then, the measurement was compared to a calibration curve of catechin (CT) and the results were expressed as milligrams of catechin equivalents (CTE) per gram of tea [mg CTE/g].

2.2.4. Antioxidant assays of the tea extracts

The free radical scavenging evaluated by the DPPH assay was determined in triplicate using the method proposed by Brand-Williams, Cuvelier, and Berset (1995), with minor changes. Firstly, to every diluted tea extract (1:11) (100 μL) was added 3.9 mL of a 125 μmol/L methanolic DPPH solution. The absorbance at a wavelength of 517 nm was measured using a spectrophotometer (Ultrospec 1100 Pro, Biomicron Ltd., Cambridge, England) after the solution had been allowed to stand in the dark for 30 min. A standard curve of Trolox (100–1000 μmol/L) was plotted and the results were expressed in mmoL Trolox equivalent per g of tea [mmol TE/g].

ABTS scavenging activity of tea extracts was determined in triplicate using the method describe by Re et al. (1999), with minor modifications. Firstly, the stock solutions of 7 mmol/L ABTS solution and 2.45 mmol/L of potassium persulfate solution were prepared. The working solution was then prepared by mixing 3 mL of each stock solution and allowing them to react for 16 h at room temperature (25 °C) in the dark. The solution was then diluted by mixing 4.0–4.5 mL ABTS radical cation solution with 250 mL distilled water to obtain an absorbance of 0.70 at 734 nm. To diluted tea extracts (1:100) (100 μL) was added 1700 μL of the $ABTS^{\bullet+}$ solution in amber centrifuge tubes. The mixture was vortexed and stored in the dark for 30 min, and the absorbance at 734 nm was measured. The results were compared a standard curve (Trolox 100–1000 μmol/L) and expressed in mmoL Trolox equivalent per g of tea [mmol TE/g].

The total antioxidant potential of the tea extracts was performed using the ferric reducing antioxidant power (FRAP) assay according to the method described by Benzie and Strain (1996), with minor changes. The FRAP reagent was prepared by a mixture of acetate buffer (300 mmol/L, pH 3.6), a solution of 10 mmol/L of TPTZ in 40 mmol/L of HCl, and 20 mmol/L of $FeCl_3$ at 10:1:1 (v/v/v). Then, 3.0 mL of the freshly prepared FRAP reagent and 100 μL of diluted extracts (1:25) were added in test tubes and mixed for 10 s. Measurements were performed using the spectrophotometer at 593 nm after 30 min in the dark. The absorbance were compared a standard curve (Trolox 100–1000 μmol/L) and results expressed in mmoL Trolox equivalent per g of tea [mmol TE/g]. All determinations were performed in triplicate.

The oxygen radical absorbance capacity (ORAC) assay was performed to measure the peroxyl radical-scavenging activity of extracts based on the method described by Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002). Firstly, the extracts were diluted (1:500) in 75 mmol/L phosphate buffer (pH 7.4). Then, a Precision 2000 automated microplate pipetting system (BIO-TEK Instruments, Inc., Winooski, VT) was used for plate-to-plate transfer of solutions. For analysis, a FLx 800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) was used with fluorescence filters for an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm programmed to record the fluorescence every minute after the addition of AAPH (153 mmol/L in 75 mmol/L phosphate buffer, pH 7.4) for 50 min,

Table 1
Total phenolics, total flavonoids and antioxidant assays from white tea extracts.

Assay	Time (min)	Temperature (°C)	Ethanol (%)	TPC (mg GAE/g)	TFC (mg CE/g)	DPPH (mmol TE/g)	ABTS (mmol TE/g)	FRAP (mmol TE/g)	ORAC (mmol TE/g)
1	5 (−1)	30 (−1)	20 (0)	37.57 ± 2.08	18.01 ± 0.13	759.19 ± 37.76	1116.69 ± 12.50	588.51 ± 10.01	1544.55 ± 47.54
2	15 (1)	30 (−1)	20 (0)	59.45 ± 1.15	21.80 ± 0.52	844.37 ± 28.00	1427.14 ± 6.44	720.10 ± 16.37	1756.26 ± 96.16
3	5 (−1)	70 (1)	20 (0)	83.04 ± 1.36	27.38 ± 0.26	990.77 ± 19.16	1606.59 ± 11.71	917.33 ± 13.56	2107.26 ± 116.45
4	15 (1)	70 (1)	20 (0)	96.62 ± 2.91	29.78 ± 0.58	1005.02 ± 26.27	1577.72 ± 12.50	927.40 ± 23.55	2013.78 ± 79.88
5	5 (−1)	50 (0)	10 (−1)	72.55 ± 3.28	20.49 ± 0.38	850.43 ± 18.67	1340.50 ± 12.88	726.01 ± 5.74	1484.51 ± 20.66
6	15 (1)	50 (0)	10 (−1)	84.02 ± 1.29	22.46 ± 0.61	858.92 ± 21.73	1341.53 ± 5.36	755.52 ± 14.09	1694.87 ± 51.06
7	5 (−1)	50 (0)	30 (1)	94.51 ± 2.64	26.59 ± 0.62	934.09 ± 27.19	1459.11 ± 18.82	846.84 ± 19.94	1894.11 ± 37.70
8	15 (1)	50 (0)	30 (1)	100.12 ± 1.29	29.61 ± 0.30	1013.20 ± 14.39	1413.73 ± 6.44	996.15 ± 21.14	2087.93 ± 99.28
9	10 (0)	30 (−1)	10 (−1)	71.49 ± 1.66	17.74 ± 0.28	735.25 ± 8.08	1042.44 ± 21.06	730.17 ± 13.59	1548.86 ± 134.98
10	10 (0)	70 (1)	10 (−1)	95.00 ± 1.12	21.24 ± 0.07	885.59 ± 26.70	1595.25 ± 19.32	951.01 ± 12.86	1947.48 ± 175.51
11	10 (0)	30 (−1)	30 (1)	76.94 ± 2.00	18.46 ± 0.40	832.24 ± 9.81	1401.35 ± 25.01	883.65 ± 8.90	1745.04 ± 127.27
12	10 (0)	70 (1)	30 (1)	106.95 ± 2.58	26.98 ± 0.45	1028.96 ± 18.57	1499.33 ± 8.19	981.56 ± 10.05	2100.25 ± 100.33
13	10 (0)	50 (0)	20 (0)	97.93 ± 1.71	21.19 ± 0.72	922.57 ± 14.12	1533.37 ± 8.19	907.95 ± 12.60	1993.16 ± 112.99
14	10 (0)	50 (0)	20 (0)	106.87 ± 2.49	21.98 ± 0.25	948.03 ± 8.33	1571.53 ± 4.73	927.05 ± 17.72	1933.08 ± 117.71
15	10 (0)	50 (0)	20 (0)	114.27 ± 1.60	21.19 ± 0.43	929.85 ± 12.30	1484.89 ± 11.71	922.53 ± 26.73	2035.61 ± 9.85
*P (Hartley)				0.97	0.59	0.82	0.64	0.94	0.28
**P (*one-way* ANOVA)				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Note: Results expressed as mean ± standard deviation. TPC: total phenolic compounds, TFC: total flavonoids content, *in vitro* antioxidant activity by DPPH: 1,1-diphenyl-2-picrylhydrazyl, ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), FRAP: ferric reducing antioxidant power, ORAC: oxygen radical absorbance capacity. * Probability values obtained by Hartley test (*F*-max) for homogeneity of variances; ** Probability values obtained by one-way ANOVA.

and the area under the curve of the fluorescence decay was integrated using software KC4 3.0. Each tea extract was measured three times, and results were expressed as mmol Trolox equivalents per gram [mmol TE/g].

2.2.5. HPLC-DAD analysis of EGCG, ECG, EC, and catechin

The HPLC analysis was performed in triplicate according the method proposed by Zielinski et al. (in press), with minor changes. Firstly, tea extracts were filtered through a 0.45 μm nylon syringe filter prior to analysis and 10 μL of sample were injected in a high performance liquid chromatography (model 2695 Alliance, Waters, Milford, MA, USA) coupled with Waters 2996 photodiode array detector (Waters, Milford, MA, USA), quaternary pump and an auto sampler. The separation was carried out using a Gemini® C18 column with dimensions of 4.6 mm × 150 mm, 5.0 μm (Phenomenex, Torrance, CA, USA) at 25 °C. The mobile phase consisted of A (0.1% acetic acid, v/v) and B (acetonitrile) with the flow rate of 0.8 mL/min. A linear gradient was programmed as follows: 0–5 min, 3–9% B, 5–15 min, 9–16% B, and 15–33 min, 16–36.4% B, followed by washing and reconditioning of the column. Identification of flavan-3-ols was performed by comparing their retention time and spectra with those of reference standards (EGCG, ECG, EC, and catechin) and quantification was performed by external calibration curves and the results expressed in mg/g. All analysis was performed in triplicate.

2.2.6. HPLC–DAD–Q-TOF–MS/MS

The elution from HPLC, obtained using the same conditions as described above in Section 2.2.5, was introduced into the mass spectrometer (Q-TOF MS) (Micromass, Waters, Milford, MA, USA) using electrospray ionization (ESI) in negative mode. Firstly, Q-TOF-MS was calibrated using sodium iodide for the negative mode through the mass range of 100–1000. Then, full mass spectra of the sample was acquired using a capillary voltage of 1.2 kV and cone voltage of 35 V. The flow rate of cone gas was 50 L/h and desolvation gas flow was 900 L/h. The desolvation gas temperature and the ion source temperature were 250 °C and 120 °C, respectively. The MS/MS spectra were acquired by using collision energy of 30 V.

2.2.7. Statistical analysis

All data were presented as mean ± standard deviation (SD). Firstly, the variables were checked for normality (Shapiro–Wilk's test) and homogeneity of variances (Hartley's test). As all variables showed normal distribution and homogeneity of variance, considering $P \geq 0.05$, then *one-way* ANOVA was performed to detect significant differences among the tea extracts for each dependent variable. Pearson correlation coefficient (r) was used to evaluate the strength between two continuous variables among the variables evaluated. A p-value below 0.05 was considered significant.

In the order to model the extraction of phenolic antioxidants from white tea, response surface methodology (RSM) coupled with multiple linear regression was used. Thus, a second-order polynomial equation was used to fit the experimental data. The generalized model used in the RSM is shown in Equation (1):

$$Y_n(x) = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_{ii}^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} X_i X_j$$

where Y_n is the predicted response, b_0, b_i, b_{ii}, and b_{ij} are the regression coefficients for linear, quadratic and interaction terms, respectively, and X_i, and X_j are the independent variables. The statistical significance of the equations was examined by ANOVA for each response, where the terms that were not significant were removed from the model and the data were re-fitted only to the significant parameters ($P < 0.05$) and the surfaces were constructed. The adequacy and quality of fitting were evaluated by $P_{lack\text{-}of\text{-}fit}$, determination coefficient (R^2) and their adjusted R^2.

Once the mathematical models were obtained, the multi-response optimization using desirability function proposed by Derringer and Suich (1980) was used to maximize the antioxidant activity (DPPH, ABTS, and FRAP) and the individual content of EGCG and ECG. In order to verify the adequacy of models, an external validation was conducted at the optimum point suggested and the result was evaluated by absolute errors (%) and predicted intervals to a level of 95%. All statistical analyses were performed using Statistica 7.0 (StatSoft Inc., USA).

3. Results and discussion

3.1. Composition of the phenolics and in vitro antioxidant activity

The mean values of the tea extracts are shown in Tables 1 and 2. All variables varied significantly ($P < 0.001$), where the total

Table 2
LC-DAD of individual catechins from white tea extracts.

Assay	Time (min)	Temperature (°C)	Ethanol (%)	(+)-Catechin (mg/g)	(−)-Epicatechin (mg/g)	EGCG (mg/g)	ECG (mg/g)
1	5 (−1)	30 (−1)	20 (0)	2.23 ± 0.24	6.34 ± 1.07	13.45 ± 0.40	3.49 ± 0.62
2	15 (1)	30 (−1)	20 (0)	2.46 ± 0.15	6.67 ± 0.71	19.19 ± 1.41	5.50 ± 0.71
3	5 (−1)	70 (1)	20 (0)	3.89 ± 0.02	10.26 ± 1.02	55.23 ± 0.96	11.61 ± 0.09
4	15 (1)	70 (1)	20 (0)	4.39 ± 0.17	9.23 ± 0.42	43.53 ± 2.31	13.45 ± 0.11
5	5 (−1)	50 (0)	10 (−1)	3.71 ± 0.18	10.35 ± 0.24	33.39 ± 1.82	8.49 ± 0.03
6	15 (1)	50 (0)	10 (−1)	2.47 ± 0.20	7.36 ± 0.11	24.18 ± 0.25	6.61 ± 0.09
7	5 (−1)	50 (0)	30 (1)	3.83 ± 0.45	11.62 ± 0.46	40.22 ± 2.43	11.94 ± 0.74
8	15 (1)	50 (0)	30 (1)	5.67 ± 0.39	15.01 ± 1.73	58.96 ± 3.14	18.39 ± 0.31
9	10 (0)	30 (−1)	10 (−1)	1.93 ± 0.01	5.71 ± 0.56	14.67 ± 0.48	3.89 ± 0.16
10	10 (0)	70 (1)	10 (−1)	2.80 ± 0.13	7.67 ± 0.35	30.22 ± 1.44	8.81 ± 0.38
11	10 (0)	30 (−1)	30 (1)	4.28 ± 0.31	12.02 ± 1.00	42.16 ± 0.84	12.16 ± 0.70
12	10 (0)	70 (1)	30 (1)	6.68 ± 0.10	18.36 ± 0.44	68.46 ± 8.62	22.85 ± 0.05
13	10 (0)	50 (0)	20 (0)	5.85 ± 0.23	15.69 ± 0.51	52.49 ± 5.96	16.69 ± 0.51
14	10 (0)	50 (0)	20 (0)	6.05 ± 0.38	13.83 ± 1.40	53.46 ± 1.41	15.16 ± 0.79
15	10 (0)	50 (0)	20 (0)	5.71 ± 0.39	13.18 ± 0.36	45.14 ± 1.21	12.67 ± 0.10
*P (Hartley)				0.61	0.85	0.25	0.36
**P (*one-way* ANOVA)				<0.001	<0.001	<0.001	<0.001

Note: Results expressed as mean ± standard deviation. EGCG: (−)-epigallocatechin gallate, ECG: (−)-epicatechin gallate. * Probability values obtained by Hartley test (*F*-max) for homogeneity of variances; ** Probability values obtained by one-way ANOVA.

phenolics and total flavonoids range from 38 to 114 mg/g and from 18 to 30 mg/g, respectively. The individual catechins determined by HPLC varied from 14 to 75 mg/g (EGCG), 3.5–22.9 mg/g (ECG), 5.7–18.4 mg/g (EC), and 1.9–6.7 mg/g (catechin). In the work performed by Socha et al. (2013), the flavan-3-ols in white tea showed similar results with our study, ranging from 1.2 to 2.5 mg/g (catechin), 1.7–5.6 mg/g (EC), 25–75 mg/g (EGCG), 11.8–24.4 mg/g (ECG), and 5.9–8.4 mg/g (EGC). Our results are also in agreement with the study performed by Unachukwu et al. (2010) in the evaluation of TPC and catechins using two different solvents (water and methanol) in the extraction of phenolics from white teas. Wang et al. (2000) established that the content of flavan-3-ols found in unfermented teas are in the following order (−)-EGCG > (−)-EGC > (−)-EC > (−)-ECG > (+)-C.

The antioxidant activity showed a large variation among the assays used: DPPH range from 735 to 1029 mmol/g, ABTS from 1042 to 1607 mmol/g, FRAP from 589 to 996 mmol/g and ORAC from 1485 to 2107 mmol/g (Table 1). The variation among the results found by *in vitro* antioxidant assays is due to different mechanisms that are involved in the determination. DPPH and ABTS radical cations are two stable and colored free radicals that have the same mechanism, in which a solution reactant is mixed with the tea extract that can donate a hydrogen atom, and the reduced form of the radical is generated followed by loss of color (Ali et al., 2008). On the order hand, FRAP is characterized by electron transfer ability, that result in the reduction of iron ions in the presence of antioxidants compounds (Craft, Kerrihard, Amarowicz, & Pegg, 2012). Lastly, the ORAC test evaluate the ability of the antioxidant compounds to inhibit the consumption of the target molecule mediated by peroxyl radical (for example, AAPH) (López-Alarcón & Denicola, 2013).

Using Pearson's correlation, it was possible to verify that total phenolic compounds and total flavonoids had a significant ($P < 0.03$) correlation with antioxidant activity measured by DPPH ($r = 0.761$; $r = 0.879$, respectively), ABTS ($r = 0.682$; $r = 0.567$, respectively), FRAP ($r = 0.884$; $r = 0.607$, respectively), and ORAC ($r = 0.772$; $r = 0.724$, respectively). A significant correlation ($P < 0.01$) also was observed among all antioxidant methods evaluated showing a correlation coefficient higher than $r > 0.77$. The flavan-3-ols stand out as main components with antioxidant power in teas, with their antioxidant power being related to the galloyl and hydroxyl groups present in their structure (Aron & Kennedy, 2008). In accordance with our study, all individual catechins showed significant correlations ($P < 0.03$) with *in vitro* antioxidant assays, with the exception of epicatechin that did not correlate with ABTS ($r = 0.497$, $P = 0.06$).

3.2. Multiple linear regression coupled with RSM

With the aim of finding the best conditions to perform an adequate extraction, response surface methodology coupled with multiple regression analysis has been used. Bae, Ham, Jeong, Kim, and Kim (2015) used a central composite design (CCD) with 5 levels and 3 factors and RSM to obtain the best conditions of extraction for different kinds of teas (green, oolong, black, and mate) based on the content of total phenolic compounds. In another study with a similar goal, Ghoreishi and Heidari (2013) performed the optimization of the extraction of epigallocathechin-3-gallate from green tea by supercritical fluid technology using central composite rotatable design (CCDR) with four independent variables.

According Granato, Gravink, Zielinski, Nunes, and van Ruth (2014), multiple linear regression associated to RSM aims to understand the data obtained into an equation in the form that explains the phenomena under investigation. Therefore, all models proposed by multiple regression analysis for the antioxidant variables were significant ($P < 0.001$), did not present lack of fit ($P > 0.05$) and could explain up to more than 85% of all variance in data with adjusted $R^2 > 0.80$, with exception for ORAC ($R^2 = 0.736$ and $R^2_{Adj} = 0.692$) (Table 3). In relation to the effects of models, all linear effects contributed positively and significantly ($P < 0.05$) in the extraction, while quadratic regression coefficients and interaction among linear *vs.* linear or quadratic *vs.* linear coefficients varied according to the dependent variable studied. For example, for antioxidant activity by ABTS assay, the quadratic regression coefficient of ethanol concentration (X_3), interactions of time (X_1) *vs.* temperature (X_2) and temperature (X_2) *vs.* ethanol concentration (X_3) showed a significantly negative effect in the extraction. The effects of parameters on dependent variables are shown in 3D-response surface plots (Fig. 1A–J) as function of time and temperature.

According to Spigno, Tramelli, and De Faveri (2007), time and temperature are important to minimize energy costs of the process. In addition the use of temperature increases the solubility of solute

Table 3
Effects of independent variables (time, temperature, and ethanol concentration) for different dependent variables evaluated.

Response variable	Factors	Regression coefficient	Standard error	*t*-value	*p*-value	−95% confidence	+95% confidence
Total phenolic compounds (mg/g)	*Constant*	106.32	3.09	34.42	<0.001	99.33	113.30
	x_1	6.57	2.27	2.89	0.02	1.43	11.71
	x_1^2	−18.49	3.34	−5.54	<0.001	−26.03	−10.94
	x_2	17.02	2.27	7.49	<0.001	11.88	22.16
	x_2^2	−18.69	3.34	−5.60	<0.001	−26.24	−11.14
	x_3	6.93	2.27	3.05	0.01	1.79	12.08
	R^2	0.9360					
	adjusted R^2	0.9005					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.79					
Total flavonoids (mg/g)	*Constant*	21.25	0.50	42.16	<0.001	20.13	22.38
	x_1	1.40	0.47	2.96	0.01	0.35	2.45
	x_1^2	3.26	0.69	4.73	<0.001	1.73	4.80
	x_2	3.67	0.47	7.78	<0.001	2.62	4.72
	x_3	2.46	0.47	5.22	<0.001	1.41	3.51
	R^2	0.9225					
	adjusted R^2	0.8914					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.09					
DPPH (mmol TE/g)	*Constant*	922.44	9.27	99.47196	<0.001	901.7793	943.104
	x_1	23.38	8.67	2.69499	0.02	4.0497	42.7055
	x_2	92.41	8.67	10.65329	<0.001	73.0836	111.7394
	x_2^2	−37.27	12.70	−2.93482	0.02	−65.5598	−8.9735
	x_3	59.79	8.68	6.89253	<0.001	40.4611	79.1169
	R^2	0.9465					
	adjusted R^2	0.9251					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.21					
ABTS (mmol TE/g)	*Constant*	1473.99	25.74	57.26	<0.001	1415.76	1532.22
	x_2	161.41	24.08	6.70	<0.001	106.94	215.88
	x_3	56.73	24.08	2.36	0.04	2.25	111.20
	x_3^2	−87.34	35.25	−2.48	0.04	−167.07	−7.60
	x_1x_2	−84.83	34.05	−2.49	0.03	−161.87	−7.80
	x_2x_3	−113.71	34.05	−3.34	0.01	−190.74	−36.67
	R^2	0.8915					
	adjusted R^2	0.8313					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.28					
FRAP (mmol TE/g)	*Constant*	922.32	5.20	177.48	<0.001	907.89	936.75
	x_1	40.06	3.82	10.47	<0.001	29.44	50.68
	x_1^2	−93.55	5.61	−16.67	<0.001	−109.13	−77.97
	x_2	79.69	5.41	14.73	<0.001	64.67	94.70
	x_2^2	−38.08	5.61	−6.78	<0.001	−53.67	−22.50
	x_3	46.01	5.41	8.51	<0.001	30.99	61.02
	x_1x_2	−30.38	5.41	−5.62	<0.001	−45.40	−15.36
	$x_1^2x_2$	54.34	7.65	7.10	<0.001	33.10	75.58
	x_1x_3	29.95	5.41	5.54	0.01	14.93	44.97
	$x_1^2x_3$	44.36	7.65	5.80	<0.001	23.12	65.60
	x_2x_3	−30.73	5.41	−5.68	<0.001	−45.75	−15.71
	R^2	0.9977					
	adjusted R^2	0.9918					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.43					
ORAC (mmol TE/g)	*Constant*	1859.12	30.75	60.45	<0.001	1792.11	1926.13
	x_2	196.76	42.11	4.67	<0.001	105.00	288.51
	x_3	143.95	42.11	3.42	0.01	52.20	235.70
	R^2	0.7364					
	adjusted R^2	0.6924					
	P-value (model)	<0.01					
	P-value (lack-of-fit)	0.15					
EGCG (mg/g)	*Constant*	51.60	3.31	15.578	<0.001	44.22	58.985
	x_1^2	−11.55	3.58	−3.228	0.01	−19.52	−3.577
	x_2	12.36	2.44	5.069	<0.001	6.93	17.791
	x_2^2	−10.55	3.58	−2.948	0.02	−18.52	−2.578
	x_3	14.72	2.44	6.036	<0.001	9.28	20.148
	R^2	0.8889					
	adjusted R^2	0.8444					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.56					
ECG (mg/g)	*Constant*	14.82	1.07	13.91	<0.001	12.45	17.19
	x_1^2	−3.45	1.15	−2.99	0.01	−6.01	−0.88
	x_2	3.96	0.78	5.05	<0.001	2.21	5.71
	x_2^2	−2.88	1.15	−2.50	0.03	−5.44	−0.31

(*continued on next page*)

Table 3 (*continued*)

Response variable	Factors	Regression coefficient	Standard error	*t*-value	*p*-value	−95% confidence	+95% confidence
	x_3	4.69	0.78	5.98	<0.001	2.95	6.44
	R^2	0.8831					
	adjusted R^2	0.8364					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.52					
EC (mg/g)	*Constant*	14.13	0.80	17.69	<0.001	12.35	15.91
	x_1^2	−2.97	0.86	−3.44	0.01	−4.89	−1.05
	x_2	1.85	0.59	3.15	0.01	0.54	3.16
	x_2^2	−3.11	0.86	−3.61	0.01	−5.04	−1.19
	x_3	3.24	0.59	5.51	<0.001	1.93	4.55
	R^2	0.8639					
	adjusted R^2	0.8095					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.41					
Catechin (mg/g)	*Constant*	5.48	0.297	18.46	<0.001	4.81	6.150
	x_1^2	−1.27	0.320	−3.95	0.01	−1.99	−0.541
	x_2	0.86	0.218	3.93	0.01	0.36	1.351
	x_2^2	−1.26	0.320	−3.94	0.01	−1.99	−0.537
	x_3	1.19	0.218	5.46	<0.001	0.70	1.686
	x_1x_3	0.77	0.309	2.50	0.03	0.07	1.471
	R^2	0.8995					
	adjusted R^2	0.8436					
	P-value (model)	<0.001					
	P-value (lack-of-fit)	0.06					

and the diffusion coefficient. Nevertheless, it is inevitable that an intense increase in temperature can cause the loss of phenolic compounds. The polarity plays an important role in the extraction of phenolic compounds of plant material. The alcoholic solvents are among the solvents commonly used. In fact, alcohols and polar solvents extract flavonoids and tannins from raw plants (Dai & Mumper, 2010). According Spigno et al. (2007) the mixtures between water and alcohols have been more efficient in the extraction of phenolic compounds in comparison to the mono-component solvent system. Methanol and ethanol are the principal alcohols used in the extraction although ethanol has advantages regarding its safety for human consumption.

Other studies also have shown the effect these or others variables pose in the extraction of bioactive compounds. Alberti et al. (2014) in their study involving extraction of phenolic compounds from apple were able to verify that the process condition, as well as the type of solvent and concentration influenced significantly the levels of compounds extracted. Using ultrasonic-assisted extraction, Yan, Yu, Chen, Li, and Li (2011) observed the effect the ratio of water to raw material (solute/solvent), extraction time and extraction temperature in the extraction of polysaccharides from *Tremella mesenterica*.

3.3. Optimization and verification of predictive models

After modeling the extraction of phenolic antioxidants of the white tea, the multi-response optimization procedure using the desirability function (D) was conducted with the models in order to maximize the antioxidant activity and individual compounds measured by HPLC. The optimum condition for this optimization suggested with a d = 0.9611, which means that 96% of the proposed aim was found by the optimization in which the extraction occurring with 30% of ethanol for 10 min at 66 °C. For the purpose of verifying the optimum condition, an external validation was performed, and the observed and predicted values with the computed absolute errors (AE) in the extraction were: DPPH (mmol/g) (observed: 994.10 ± 5.36, predicted: 1032.31, AE = 3.84%), ABTS (mmol/g) (observed: 1582.55 ± 17.78, predicted: 1481.54, AE = 6.38%), FRAP (mmol/g) (observed: 964.10 ± 11.24, predicted: 983.12, AE = 1.96%), EGCG (mg/g) (observed: 69.57 ± 0.94, predicted: 69.42, AE = 0.17%), and ECG (mg/g) (observed: 20.47 ± 0.02, predicted: 20.84, AE = 1.82%). All the observed values were within the predicted interval to a level of 95%. Therefore, the models could be used to predict the response values.

Due to interest by industries and consumers in products supplemented with tea extracts, application of predominantly green tea extracts have been studied in the meat products, active films, oils and fats, beverages, bakery products, etc. (Senanayake, 2013). However, we would like to emphasize that white tea as well as green tea can be a good source to supplement food products with bioactive compounds. Future studies should focus on the application and evaluation of white tea extracts in food products and to perform comparisons with other vegetable/tea extracts in related studies.

3.4. Identification of phenolic compounds by LC-DAD-MS/MS under optimum conditions

The phenolic profile of the extract was identified in the best condition on the basis of their LC retention time, wavelength and mass spectra based on the values of mass-to-charge ratio (*m/z*) and comparisons with fragmentation patterns. The chromatogram of phenolic compounds analyzed is shown in Fig. 2.

The phenolic acids identified in the white tea extract were gallic acid (peak 1, t_R = 7.15 min, λ_{MAX} = 271 nm) and 5-galloylquinic acid (peak 2, t_R = 8.30 min, λ_{MAX} = 274 nm) which produced the $[M-H]^-$ ions at *m/z* 169 and 343 and mass fragment ions at *m/z* 125 and 191 (only for 5-galloylquinic acid). These compounds have been reported among the principal phenolic acids present in *Camellia sinensis* tea (Wu, Xu, Héritier, & Andlauer, 2012), and theobromine (peak 3, t_R = 9.05 min, λ_{MAX} = 273 nm) and caffeine (peak 6, t_R = 14.28 min, λ_{MAX} = 273 nm) as the principal methylxanthines (confirmed using standards reference). According to Pinto (2013), tea consumption has been associated with positive effects on blood pressure, cognitive performance, mood, and on sleep.

Gallocatechin (peak 4, t_R = 10.17 min, λ_{MAX} = 270 nm) and epigallocatechin (peak 5, t_R = 19.45 min, λ_{MAX} = 270 nm) showed

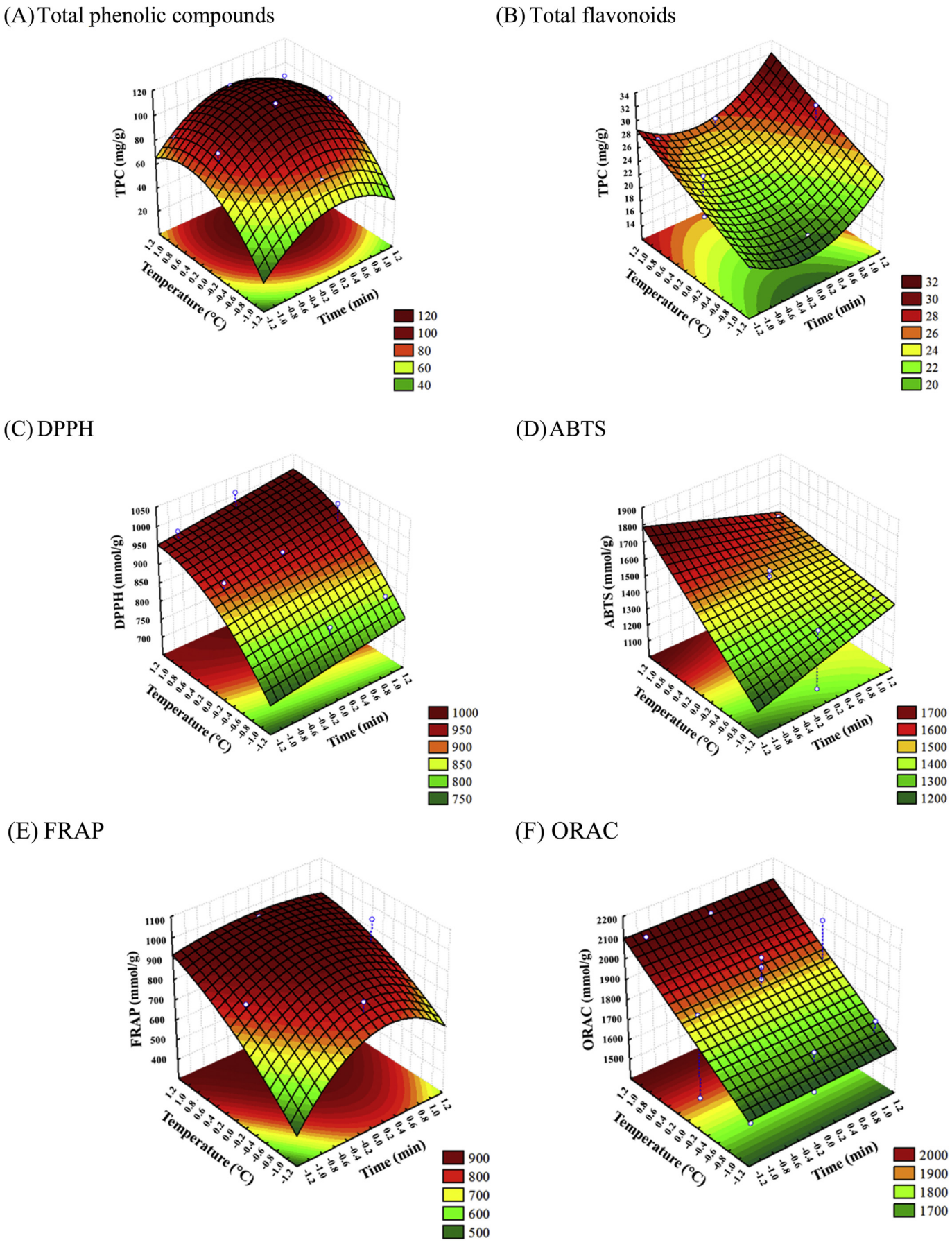

Fig. 1. Response surface plots of the effects of temperature, time and ethanol concentration.

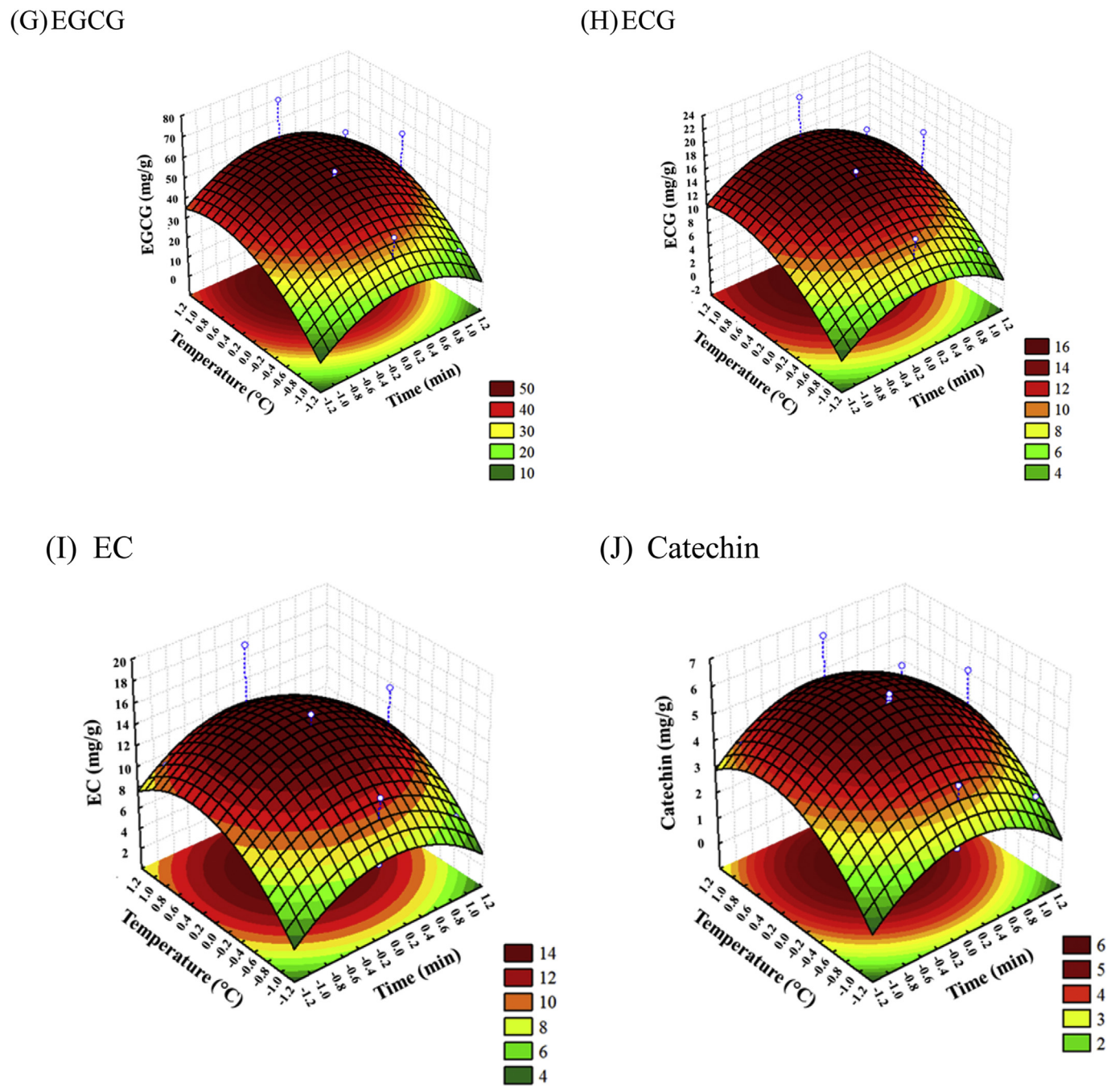

Fig. 1. (*continued*).

the [M−H] − ion at *m/z* 305 with the same mass fragments ions at *m/z* 167, 137, and 125 (Fig. 2-B). Catechin (peak 7, t_R = 15.57 min, λ_{MAX} = 278 nm) and epicatechin (peak 8, t_R = 18.43 min, λ_{MAX} = 278 nm) also had the same [M−H] − ion at *m/z* 289 and the same fragments ions at *m/z* 109, 121, 191, 205, and 245. Epigallocatechin gallate (peak 9, t_R = 19.45 min, λ_{MAX} = 274 nm) showed [M−H] − ion at *m/z* 457 and fragments ions at *m/z* 305, 169, and 125, while epicatechin gallate (peak 10, t_R = 24.28 min, λ_{MAX} = 277 nm) presented [M−H] − ion at *m/z* 441 and fragments ions at *m/z* 289, 169, and 125 (Fig. 2-C). The identification of phenolic compounds (catechins and phenolic acids) was performed according to Del Rio et al. (2004) and Wu et al. (2012) who identified phenolic compounds present in tea at different fermentation stages (non-fermented to fully fermented).

4. Conclusion

A Box-Behnken design was applied to evaluate the extraction of phenolic compounds and *in vitro* antioxidant activity from white tea. Using response surface methodology coupled with multiple regression, it was possible to suggest mathematical models to evaluate dependent variables. Multiple response optimization using DPPH, ABTS, FRAP, EGCG, and ECG was effective with 96% of the proposed aim targeted with the best combinations of the variables for increasing the yield of the extraction, where the optimum point was found at 10 min, 66 °C, and 30% of ethanol. Therefore, white tea

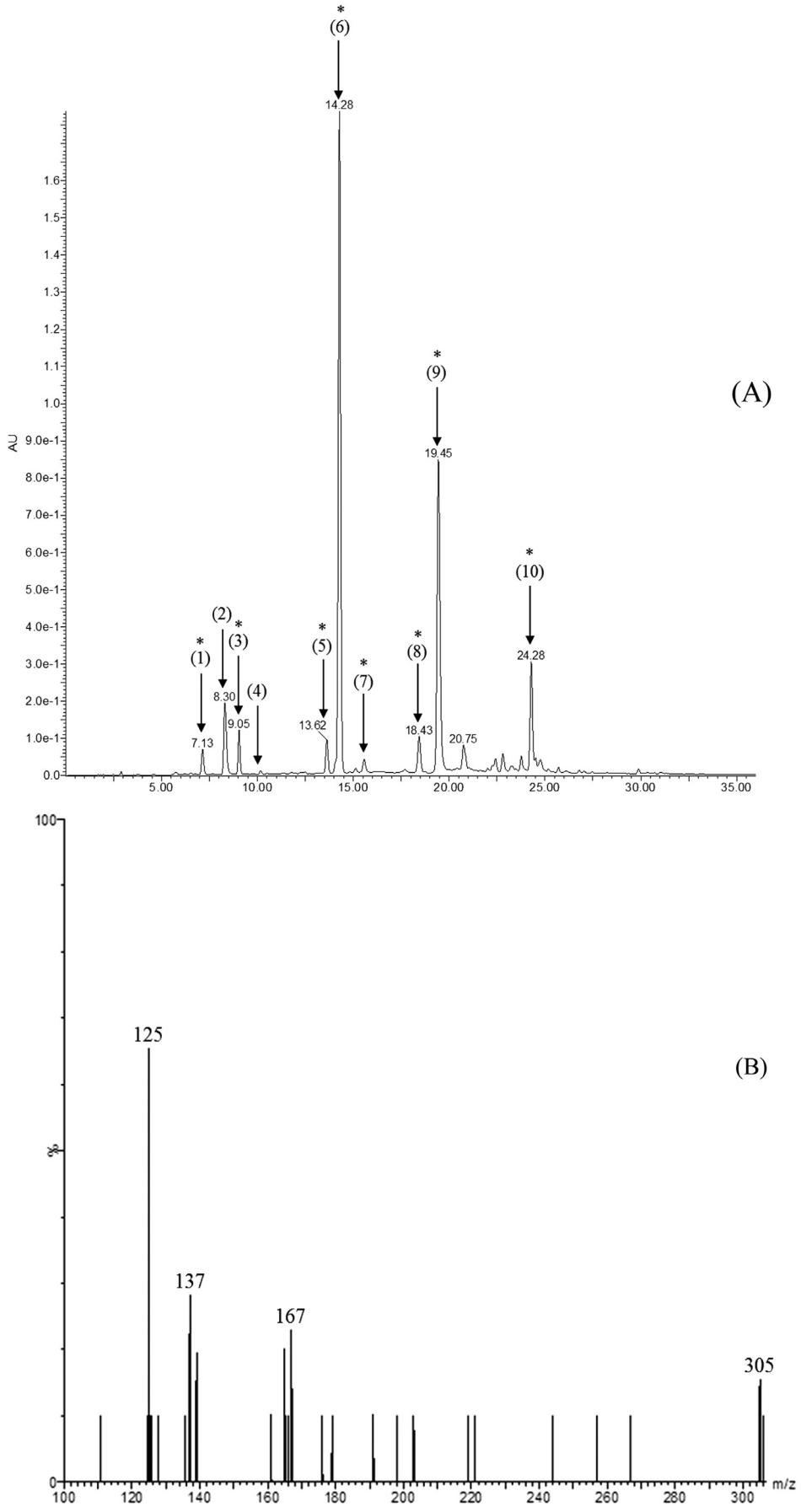

Fig. 2. Chromatogram of white tea extract obtained at the optimum point (10 min, 66 °C, 30% of ethanol) at 280 nm (A), mass spectra for GC and EGC (B), and ECG (C). Note: (1) gallic acid, (2) 5-galloylquinic acid, (3) theobromine, (4) gallocatechin, (5) epigallocatechin, (6) caffeine, (7) catechin, (8) epicatechin, (9) epigallocatechin gallate, (10) epicatechin gallate. *samples identified and confirmed using standard of reference.

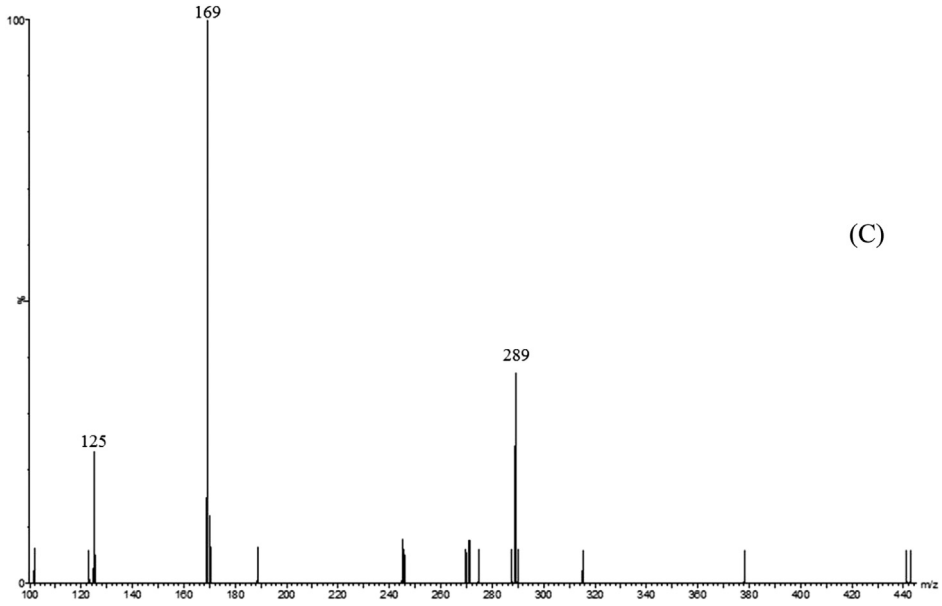

Fig. 2. (*continued*).

extract can be a good resource of bioactive compounds (principally flavan-3-ols) to supplement food products.

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