Optimization of antioxidants extraction from soybeans fermented by *Aspergillus oryzae*

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ABSTRACT

The extraction of antioxidant compounds from soybeans fermented with *Aspergillus oryzae* was optimised using a factorial design. A kinetic study of the total phenolic production and DPPH scavenging activity was first performed at the points selected in the factorial design. In both cases, the experimental profiles were fitted to a modified first-order kinetic model. To investigate the combined effects of temperature and solvent concentration on the extraction, the parameters obtained from the fitted kinetic models were used as response variables in a rotatable second-order design with quintuple replications in the centre of the experimental domain. The results obtained indicate that temperature had the most significant effect. The response surfaces show a maximum in the experimental domain studied. The optimum conditions for the extraction of total phenolic content were 65.3^{o}C and 73.1% ethanol, in which 56.2 mg of GAE/g were predicted. A scavenging activity of 81.6% DPPH was predicted at the optimum conditions of 61.6^{o}C and 60% ethanol.

Keywords: Total phenolic; DPPH; soybean; *Aspergillus oryzae*; antioxidant extraction; factorial design; kinetics optimization.

INTRODUCTION

Antioxidant compounds play an important role in human health. A diet rich in foods containing molecules with antioxidant properties can reduce the risk of human diseases (Halliwell, & Gutteridge, 1999; Tsao, & Deng, 2004). However, the growing concern about potential health hazards caused by the use of synthetic antioxidants in food products has led to the scrutiny of natural antioxidants (Wettasinghe, & Shahidi, 1999). Among these, phenolic compounds, which are widely found in plants, are the most promising group of molecules (Pratt, & Hudson, 1990; Cuppett, & Schnepf, 1997; Shahidi, & Wanasundara, 1997). Soybeans and their products are nutritionally rich foodstuff and they contain various amounts of phytochemicals (isoflavones, saponins, phytic acid, phytosterols, Kunitz and Bowman-Birk trypsin inhibitors, phenolic acids) that show functional, antioxidants and radical scavenging properties (Pratt, & Birac, 1979; Hayes, Bookwalter, & Bagley, 1977; Da Silva Pinto, Lajolo, & Genovese, 2005; Wardhani, Vázquez, & Pandiella, 2008; Isanga, & Zhang, 2008; Hubert, Berger, Nepveu, Paul, & Daydé, 2008). In some cases the antioxidant effect could be significantly enhanced through fermentation using aspergilli (Romero, Doval, Sturla, & Judis, 2004; McCue, & Shetty, 2003; Esaki, Onozaki, Kawakishi, & Osawa, 1997; Lin, Wei, & Chou, 2006).

For a practical application in the food industry antioxidants should be first extracted. The efficiency of the extraction process affects the antioxidant capacity of the extract (Hinneburg, & Neubert, 2005). Studies on the extraction of the antioxidant activity in unfermented soybeans and vine have reported a variation of the total phenolic concentration when different solvents were used, which is due to differences in their polarities (Naczk, & Shahidi, 2006; Calliste, Trouillas, Allais, Simon, & Duroux, 2001). Limited information is

66 available regarding the extraction of antioxidant compounds in fermented soybeans.
67 However, significant higher concentration of phenolics was obtained after fermentation
68 when compare to unfermented soybeans (McCue, & Shetty, 2003; Esaki, Onozaki,
69 Kawakishi, & Osawa, 1997; Lin, Wei, & Chou, 2006; Wardhani, Vázquez, & Pandiella,
70 2009). A universal extraction protocol would be difficult to establish due to the complex
71 composition of the beans and the structural diversity of the antioxidant compounds of the
72 natural source. The extraction efficiency is affected by multiple variables, amongst which
73 temperature and the nature of the solvent are the most important factors, which may act
74 dependently or independently (Liu, & Ang, 2000).
75
76 Processes are commonly optimised using one-factor-at-a-time approaches. Optimal
77 conditions or interactions between variables cannnot be predicted with this methodology.
78 This limitation can be overcome using experimental design methodologies (DOE; Box,
79 Hunter, & Hunter, 1989; Akhnazarova, & Kafarov, 1982). DOE is a collection of statistical
80 and mathematical techniques that have been successfully used in developing, improving
81 and optimizing bio-processes (Liyana-Pathirana, & Shahidi, 2005; Juntachote, Berghofer,
82 Bauer, & Siebenhandl, 2006; Paz, Vázquez, Riobó, & Franco, 2006; Vázquez, González, &
83 Murado, 2006; Bandeira, Tininis, Bolzani, & Cavalheiro, 2006).
84
85 In this study, the optimal conditions for antioxidant extraction from soybeans fermented
86 with *Aspergillus oryzae* were investigated using two complementary and sequential
87 approaches. A factorial design was initially proposed. Kinetic analyses were then
88 performed at the temperature-ethanol concentration points of the design. The parameters
89 obtained from the fits of the kinetic data to a modified first-order model were the dependent

variables to formulate the empirical equations of the second order design. Finally, optimal conditions for a maximum antioxidant extraction were obtained from the response surfaces.

MATHERIALS AND METHODS

Microorganism

Aspergillus oryzae was originally obtained from ABM Chemicals Ltd. (Woodley, Cheshire, UK). A distilled water suspension of the fungi spores was kept at -30^{o}C until used. The volume of inoculum was 1.5 mL with a cell concentration of 1.2×10^{8} cells/mL.

Soybeans fermentation

Split soybeans (150 g) and 73.5 mL of distilled water were placed in 500 mL capped Duran bottles and autoclaved at 121^{o}C for 20 minutes. After soybeans and distilled water cooled down (at room temperature), the spore suspension was mixed with the sterile medium and the bottle was manually shaken (vertically and horizontally) for 10 minutes to homogenise the inoculum. The inoculated soybeans were poured into Petri dishes and incubated at 30^{o}C for 5 days. Soybean samples were crushed with mortar and pestle before sealed in plastic bag and store at -30^{o}C until used.

Crude phenolic extraction

Detailed extraction conditions of temperature and concentration of ethanol are shown in Table 1. Ground samples (2 g) were extracted with 20 mL of the corresponding aqueous ethanol concentration at the temperature pre-established in the factorial design using a Soxhlet System HT (1043 – Tecator). Subsequently, the extract was dehydrated to obtain a dry extract and diluted with ethanol up to 20 mg/mL of extract concentration. After that, the

114 extract was centrifugated at 16,249 × g for 5 min, and the supernatant was used for the
115 antioxidant determination.
116
117 **Determination of total phenolic content**
118 The total phenolic content was determined based on the method of Singleton, Orthofer, and
119 Lamuela-Raventós (1999), using the Folin-Ciocalteu Reagent (FCR) with gallic acid as a
120 standard. 50 μL of sample or blank were added to 3 mL of distilled water in 12 mL test
121 tubes. A volume of FCR (250 μL) was placed into the tube and mixed before adding 750
122 μL of saturated Na_2CO_3. The final volume of the reaction mixture was adjusted to 5 mL
123 with distilled water. The absorbance at 765 nm was read in 1-cm cuvettes after incubation
124 for 2 h at room temperature, and readings were compared with a standard curve of gallic
125 acid. The total phenolic content was expressed as mg of gallic acid equivalent per gram dry
126 basis of fermented soybeans (mg GAE /g db).
127
128 **Determination of DPPH radical scavenging activity**
129 The effect of the extract on 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) radical
130 was estimated according to the procedure described by Brand-Williams, Cuvelier, and
131 Berset (1995). The extract (0.1 mL) was added to 3.9 mL of DPPH 6×10^{-5} M in methanol
132 which was prepared daily. The decrease in absorbance was determined at 515 nm after
133 incubation for 30 min. A DPPH solution without sample was used as control and the DPPH
134 percentage inhibition was calculated according to the following equation:
135

136 $$DPPH\ scavenging\ effect\ (\%) = \left(1 - \frac{absorbance_{sample}}{absorbance_{control}}\right) \times 100\% \qquad (1)$$

137

138 **Experimental design and statistical analysis**

139 The antioxidant activities (total phenolic content and DPPH scavenging capacity) as a
140 function of the extraction time was studied using a rotatable second order design with
141 quintuple replication in the centre of the experimental domain (Box, Hunter, & Hunter,
142 1989; Akhnazarova, & Kafarov, 1982). The range of independent variables studied,
143 temperature (*T*) and ethanol concentration (*E*), is shown in Table 1.

144

145 The experiments were planned using two different approaches. Initially, the variation of
146 the antioxidant extraction (measured as total phenolic concentration and DPPH scavenging
147 activity) with time were fitted to appropriate mathematical models to obtain a group of
148 kinetic parameters that could describe these trends. Finally, a rotatable second order design
149 was implemented using the kinetic parameters as response.

150

151 In the first case, the calculation was carried out using a non linear least-squares (quasi-
152 Newton) method via the macro 'Solver' in the Microsoft Excel XP spreadsheet. Later, the
153 Statistica 6.0 program (StatSoft, Inc. 2001) was used to calculate the significance of the
154 estimated parameters (Student t-test, α=0.05) and the robustness of the model (Fisher *F*
155 test, α=0.05). Results of the factorial designs were employed to obtain empirical equations
156 that describe the significant parameters as a function of temperature and ethanol
157 concentration. The statistical significance of the coefficients was verified by means of the
158 Student t-test (α=0.05), and the model consistency by the Fisher *F* test (α=0.05) using the
159 following mean squares ratios:

	the model is acceptable if
F_1 = Model / Total error	$F_1 \geq F_{den}^{num}$
F_2 = (Model + Lack of fitting) / Model	$F_2 \leq F_{den}^{num}$
F_3 = Total error / Experimental error	$F_3 \leq F_{den}^{num}$
F_4 = Lack of fitting / Experimental error	$F_4 \leq F_{den}^{num}$

RESULTS AND DISCUSSION

Previous experiments using different solvents (acetone, methanol, ethanol, hexane and ethyl acetate) at various concentrations demonstrated that methanol and ethanol were the most efficient compounds in the extraction of antioxidant compounds from fermented soybeans (data not shown). Among these, ethanol was selected since it has less restrictions in food applications. Therefore, the aim of this study was to evaluate the combined effects of extraction temperature and ethanol concentration for the recovery of antioxidant compounds from fermented soybeans.

Kinetics of antioxidants activities

Kinetics of antioxidants extraction were firstly performed at the points selected in the factorial design. The results for total phenolic content and DPPH scavenging activity are shown in Figures 1 and 2. In both cases the experimental data follow hyperbolic curves, and for this reason a modified first order kinetic model with a final asymptote was chosen to describe the extraction of antioxidants with time

$$P = P_m \cdot \left(1 - e^{-k_p \cdot t}\right) \quad (2)$$

where, P is the phenolic concentration at time t (mg GAE/g db), P_m is the maximum concentration when time approaches infinite, and k_p is the specific rate of the total phenolic concentration (min^{-1}). Similarly, for the DPPH scavenging capacity

$$D = D_m \cdot \left(1 - e^{-k_d \cdot t}\right) \quad (3)$$

where, D is the DPPH scavenging activity (%), D_m the maximum DPPH scavenging activity when time approaches infinite, and k_d is the specific rate of DPPH scavenging activity (min^{-1}). The continuous curves in Figures 1 and 2 represent the models obtained by fitting the experimental data to these equations. The statistical analyses of the kinetic models are summarised in Tables 2 and 3.

In general, the proposed models were statistically robust (Fisher´s F-test and p-values < 0.001), and the parametric estimations were significant (Student´s t-test $\alpha = 0.05$). The coefficients of linear correlation (r) between predicted and observed values were in all cases higher than 0.964. This indicates that the proposed kinetic models can be used to describe and predict the extraction of antioxidants from fermented soybeans in the range of temperature and ethanol concentration assayed.

From the values of the parameters in the fitted models, it can be concluded that the highest phenolic concentration (P_m) and specific rate of total phenolic content (k_p) are found at the highest temperature studied (74ºC). The highest DPPH scavenging activity (D_m) was

achieved at the centre of the experimental domain (T=0, E=0), but the maximum specific rate of DPPH scavenging activity (k_d) is obtained at the point T=1.41 and E=0 (see Table 1).

Factorial design

As stated before, the second approach was to study the correlation between the kinetic parameters and the combined effects of temperature (T) and ethanol concentration (E). The parameters obtained from the fitted kinetic models were adjusted to the polynomic function

$$R=b_0+b_1T+b_2E+b_{12}TE+b_{11}T^2+b_{22}E^2 \quad (4)$$

where R is any of the response variables (D_m, P_m, k_p or k_d).

The best-fit model and the statistical analysis of the rotatable second-order design when R was the maximum total phenolic content (P_m) are shown in Table 4. The statistical analysis indicates that the combined term TE in equation (4) was not significant.

The response surfaces obtained from fitting the total phenolic parameters P_m and k_p to equation (4) are plotted in Figure 3. The maximum phenolic content (P_m, left) shows a well defined maximum within the experimental domain. The maximum can be calculated deriving the response equation with respect to the independent variables T and E

$$\left.\frac{\partial P_m}{\partial T}\right|_{T=T_m}=11.915-17.32T \quad \text{and} \quad \left.\frac{\partial P_m}{\partial E}\right|_{E=E_m}=2.937-6.29E$$

Since at the maximum both derivatives must be zero, it is possible to calculate the optimum temperature and ethanol concentration for a maximum antioxidant extaction; T_m = 0.690 and E_m = 0.467 in codified values, equivalent to 65.3ºC and 73.1% ethanol in real values (see Table 1 for codification/decodification). At this point the predicted maximum total phenolic concentration was 56.2 mg GAE/g db.

The best-fit model and the statistical analysis of the rotatable second-order design for the specific rate of the total phenolic concentration (k_p) are shown in Table 5. In this case the statistical analysis indicates that neither the combined term *TE* nor the *E* term in equation (4) are significant. The response for k_p (Figure 3, right) is a convex surface with a line of maxima at $E = 0$. An absolute maximum response cannot be obtained within the experimental domain. However, in all cases the specific rate of total phenolic concentration increases with temperature.

The results for maximum DPPH scavenging activity (D_m) were similar to total phenolic. Figure 4 (left) shows the parabolic response surface obtained from the equation in Table 6. Both the *E* and *TE* terms in equation (4) were not significant. The maximum can be equally calculated deriving the response equation with respect to the independent variables *T* and *E*

$$\left.\frac{\partial D_m}{\partial T}\right|_{T=T_m} = 3.35 - 8.70T \quad \text{and} \quad \left.\frac{\partial D_m}{\partial E}\right|_{E=E_m} = -13.24E$$

247 The maximum D_m was found at T_m= 0.385 (61.6ºC) and E_m= 0 (60%). At this point the
248 predicted maximum DPPH scavenging activity was 81.6%.
249
250 Table 7 summarises the results of the factorial design for the specific rate of DPPH
251 scavenging activity (k_d) plotted in Figure 4 (right). Only the combined term *TE* was not
252 significant, and the model defines a concave response surface with a line of maximum
253 slope in the proximity of E=0. As for the k_p model, a maximum response cannot be
254 calculated within the experimental domain, but k_d increases with the temperature.
255
256 The empirical models obtained show a good fitting and consistency. The correlation with
257 the observed values ($r^2_{adjusted}$) was higher that 0.85 and the experimental variability of the
258 replica in the centre of the experimental domain was considerably low, allowing for
259 construction of highly predictive models.
260
261 The improvement of the antioxidant extraction with temperature was probably due to the
262 increasing diffusivity of the solvent in the solid matrix and the solubility of the phenolic
263 compounds in the solvent, which favour the extraction (Juntachote, Berghofer, Bauer, &
264 Siebenhandl, 2006; Cacace, & Mazza, 2003; Herrero, Martin-Alvarez, Señoráns, Cifuentes,
265 & Ibáñez, 2005). However, it should be noted that increasing temperature beyond a certain
266 value can lead to decomposition of some phenolic compounds. Rostagno, Palma, and
267 Barroso (2007) reported decomposition of isoflavones in soybean during heat treatments.
268 Malonyl isoflavones also degrade when extraction is performed between 75 and 100^{o}C.
269 Extraction between 100-125^{o}C affects acetyl isoflavones and higher temperatures sharply
270 reduced the glucosides concentrations.

271

272 It is not surprising to find out that the DPPH results showed a similar trend to the total
273 phenolic concentration. However, the optimum extraction conditions were slightly different
274 for the two assays. This could be due to the fact that each assay measures different kind of
275 phenolics, and each phenolic compound shows different antioxidant properties, which
276 depends on the chemical structure and substitution position (Pokorny, 2003).

277

278 The fit of models to second-order polynomial equations was in agreement with other
279 authors who used temperature, solvent concentration and time as variables in a similar
280 approach using other food matrices. Wettasinghe, & Shahidi (1999) studied the antioxidant
281 properties of an ethanol extract of defatted borage seeds, and Herrero, Martin-Alvarez,
282 Señoráns, Cifuentes, & Ibáñez (2005) investigated antioxidants from *Spirulina platensis*
283 microalga. Liyana-Pathirana, & Shahidi (2005) studied phenolic compounds from wheat,
284 and Juntachote, Berghofer, Bauer, & Siebenhandl (2006) tested phenolic extracts of lemon
285 grass, galangal, holy basil and rosemary. However, only Herrero, Martin-Alvarez, Señoráns,
286 Cifuentes, & Ibáñez (2005) reported that temperature had the strongest influence amongst
287 all variables. In the other studies, the solvent concentration was the main factor affecting
288 antioxidant extraction. These discrepancies highlight the need for appropriate extraction
289 protocols, with suitable solvent polarity, time and temperature for each food matrix, and
290 using multivariable experimental design techniques.

291

292 **CONCLUSIONS**

293 A factorial design combined with a kinetic approach was successfully applied to maximise
294 the extraction of antioxidant compounds from soybeans fermented with *Aspergillus oryzae.*

295 The highest values of P_m and D_m were obtained close to the centre of the experimental
296 domain studied. Both k_p and k_d showed a marked increase with temperature, but absolute
297 maxima for this parameters were not predicted within the experimental domain. In general,
298 higher temperatures lead to higher yields of total phenolics and DPPH scavenging activity.
299 However, over a certain temperature value decomposition of some phenolic compounds
300 may occur. In this case, the optimal conditions for antioxidant extraction were 65.3^{o}C and
301 73.1% ethanol for maximum total phenolic concentration, and 61.6^{o}C and 60% ethanol for
302 maximum DPPH scavenging activity.
303

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439
440

FIGURE CAPTIONS

Figure 1: Kinetics of total phenolic content extracted from soybeans fermented with *Aspergillus oryzae* in each one of the experimental conditions (in natural values) defined in Table 1. The experimental data (symbols) were fitted to the model (2) (continuous line).

Figure 2: Kinetics of DPPH scavenging activity extracted from soybeans fermented with *Aspergillus oryzae* in each one of the experimental conditions (in natural values) defined in Table 1. The experimental data (symbols) were fitted to the model (3) (continuous line).

Figure 3: Response surface corresponding to the joint effect of ethanol (E) and temperature (T) on the maximum total phenolic production (P_m, left) and in the specific rates of total phenolic production (k_p, right) according to the equations described in Tables 4 and 5. Independent variables are expressed in codified values.

Figure 4: Response surface corresponding to the joint effect of ethanol (E) and temperature (T) on the maximum DPPH scavenging activity (D_m, left) and in the specific rates of DPPH scavenging activity (k_d, right) according to the equations described in Tables 6 and 7. Independent variables are expressed in codified values.

460 **TABLE CAPTIONS**

461

462 **Table 1:** Experimental domain and codification of independent variables in the factorial
463 rotatable design.

464

465 **Table 2:** Parametric estimations corresponding to the modified first order kinetic model (2)
466 applied to the extraction of total phenolic compounds from fermented soybeans by
467 *Aspergillus oryzae* at the experimental conditions studied. Independent variables are
468 expressed in natural values in brackets.

469

470 **Table 3:** Parametric estimations corresponding to the modified first order kinetic model (3)
471 applied to the extraction of DPPH scavenging activity from fermented soybeans by
472 *Aspergillus oryzae* at the experimental conditions studied. Independent variables are
473 expressed in natural values in brackets.

474

475 **Table 4:** Results of the factorial design and tests of significance for the model of maximum
476 total phenolic concentration (P_m).

477

478 **Table 5:** Results of the factorial design and tests of significance for the model of the
479 specific rate of total phenolic production (k_p).

480

481 **Table 6:** Results of the factorial design and tests of significance for the model of maximum
482 DPPH scavenging activity (D_m).

483

Table 7: Results of the factorial design and tests of significance for the model of the specific rate of DPPH scavenging activity (k_d).

TABLES

Table 1

Coded values	Natural values of temperature (T) and ethanol concentration (E)	
	T (ºC)	*E* (%)
-1.41	40	21
-1	45	32
0	57	60
+1	69	88
+1.41	74	100

Codification: $V_c=(V_n-V_0)/\Delta V_n$; Decodification: $V_n= V_0+(\Delta V_n \times V_c)$
V_n=natural value in the centre of the domain;
ΔV_n= increment of V_n per unit of V_c.
Shaded area: values corresponding to the first order design.

Table 2

Design Conditions	[a]$P_m \pm$ [b]CI	[c]$k_p \pm$ CI	[d]F (df_1=2, df_2=7; α=0.05)	p-value	[e]r (O:P)
T:-1 (45ºC); *E*:-1 (32%)	27.61 ± 12.82	0.0043 ± 0.0029	702.72	<0.0001	0.983
T: 1 (69ºC); *E*:-1 (32%)	49.15 ± 3.20	0.0142 ± 0.0023	2931.01	<0.0001	0.995
T:-1 (45ºC); *E*: 1 (88%)	33.59 ± 26.58	0.0039 ± 0.0038	305.02	<0.0001	0.964
T: 1 (69ºC); *E*: 1 (88%)	51.08 ± 2.07	0.0151 ± 0.0016	6904.27	<0.0001	0.998
T:-1.41 (40ºC); *E*: 0 (60%)	13.54 ± 3.29	0.0098 ± 0.0048	416.68	<0.0001	0.967
T: 1.41 (74ºC); *E*: 0 (60%)	53.26 ± 1.07	0.0195 ± 0.0012	20067.38	<0.0001	0.999
T: 0 (57ºC); *E*:-1.41 (21%)	38.86 ± 13.56	0.0076 ± 0.0048	345.59	<0.0001	0.966
T: 0 (57ºC); *E*: 1.41 (100%)	49.87 ± 7.75	0.0092 ± 0.0028	1160.07	<0.0001	0.988
T: 0 (57ºC); *E*: 0 (60%)	52.85 ± 5.74	0.0096 ± 0.0021	2210.10	<0.0001	0.994
T: 0 (57ºC); *E*: 0 (60%)	49.80 ± 3.32	0.0108 ± 0.0015	4580.68	<0.0001	0.997
T: 0 (57ºC); *E*: 0 (60%)	52.55 ± 5.11	0.0097 ± 0.0019	2668.48	<0.0001	0.995
T: 0 (57ºC); *E*: 0 (60%)	52.41 ± 7.53	0.0101 ± 0.0030	1118.99	<0.0001	0.988
T: 0 (57ºC); *E*: 0 (60%)	49.37 ± 5.21	0.0114 ± 0.0026	1688.54	<0.0001	0.992

[a]Maximum total phenolic concentration. [b]Confidence intervals (α = 0.05; df = 7). [c]Specific rates of total phenolic production.
[d]F-Fisher test (df_1 = degrees of freedom of the model; df_2 = degrees of freedom of the error)
[e]Correlation coefficient between observed and predicted data.

Table 3

Design Conditions	[a]$D_m \pm$ [b]CI	[c]$k_d \pm$ CI	[d]F (df_1=2, df_2=7; α=0.05)	p-value	[e]r (O:P)
T:-1 (45ºC); *E*:-1 (32%)	65.51 ± 6.11	0.0152 ± 0.0041	1124.24	<0.0001	0.992
T: 1 (69ºC); *E*:-1 (32%)	74.48 ± 3.17	0.0248 ± 0.0037	3386.46	<0.0001	0.997
T:-1 (45ºC); *E*: 1 (88%)	61.32 ± 1.03	0.0232 ± 0.0013	23578.91	<0.0001	0.999
T: 1 (69ºC); *E*: 1 (88%)	70.71 ± 3.75	0.0284 ± 0.0057	1978.03	<0.0001	0.994
T:-1.41 (40ºC); *E*: 0 (60%)	70.32 ± 6.65	0.0147 ± 0.0042	1071.03	<0.0001	0.991
T: 1.41 (74ºC); *E*: 0 (60%)	76.23 ± 2.49	0.0308 ± 0.0041	4860.86	<0.0001	0.998
T: 0 (57ºC); *E*:-1.41 (21%)	66.53 ± 4.03	0.0221 ± 0.0042	1961.18	<0.0001	0.995
T: 0 (57ºC); *E*: 1.41 (100%)	70.99 ± 3.02	0.0249 ± 0.0037	3453.07	<0.0001	0.997
T: 0 (57ºC); *E*: 0 (60%)	79.34 ± 2.76	0.0199 ± 0.0021	6576.57	<0.0001	0.999
T: 0 (57ºC); *E*: 0 (60%)	79.91 ± 2.14	0.0206 ± 0.0017	10498.72	<0.0001	0.999
T: 0 (57ºC); *E*: 0 (60%)	82.02 ± 6.11	0.0192 ± 0.0039	1569.18	<0.0001	0.994
T: 0 (57ºC); *E*: 0 (60%)	79.96 ± 5.15	0.0185 ± 0.0034	2103.97	<0.0001	0.996
T: 0 (57ºC); *E*: 0 (60%)	80.98 ± 3.70	0.0206 ± 0.0026	4099.41	<0.0001	0.997

[a]Maximum DPPH scavenging activity. [b]Confidence intervals (α = 0.05; *df* = 7). [c]Specific rates of DPPH scavenging activity.
[d]F-Fisher test (df_1 = degrees of freedom of the model; df_2 = degrees of freedom of the error)
[e]Correlation coefficient between observed and predicted data.

Table 4

T	*E*	[a] P_m	[b] $\hat{P}_m$	Coefficients from the least-squares regression	t	[c] Adjusted Model	
-1	-1	27.61	24.74	51.39	68.84	51.39	*i.t.*
1	-1	49.15	48.57	11.92	20.16	11.92	*T*
-1	1	33.59	30.61	2.94	4.97	2.94	*E*
1	1	51.08	54.44	-1.01	1.21	NS	*TE*
-1.41	0	13.54	17.38	-8.66	13.62	-8.66	T^2
1.41	0	53.26	50.98	-3.15	4.95	-3.15	E^2
0	-1.41	38.86	40.99				
0	1.41	49.87	49.28				
0	0	52.85	51.39	Average value	= 44.149		
0	0	49.80	51.39	Expected average value	= 51.396		
0	0	52.55	51.39	Var(Ee)	= 2.7867		
0	0	52.41	51.39	t(α<0.05; υ=4)	= 2.776		
0	0	49.37	51.39				

	[d] SS	[e] υ	[f] MS	[g] Mean Squares Ratios	
Model	1747.66	4	436.92	MSM/MSE= 53.99	F_8^4 (α=0.05)= 3.838
Error	64.74	8	8.092	MSMLF/MSM= 0.515	F_4^8 (α=0.05)= 6.041
Exp. Error	11.15	4	2.787	MSE/MSEe= 2.904	F_4^8 (α=0.05)= 6.041
Lack of fitting	53.59	4	13.398	MSLF/MSEe= 4.808	F_4^4 (α=0.05)= 6.388
Total	1812.40	12			

r^2=	0.964
r^2 adjusted=	0.946

[a]Experimental values of maximum total phenolic concentration. [b]Estimated values of maximum total phenolic concentration from the adjusted model. [c]Coefficients for the terms of the adjusted model: i.t., independent term; E, ethanol concentration (%); T, temperature (ºC); NS, not significant coefficient. [d]SS: sum of squares. [e]υ: degrees of freedom. [f]MS: mean squares. [g]Mean Square Ratios: MSM, mean squares of the model; MSE, mean squares for error; MSMLF, mean squares for model lack of fit; MSEe, mean squares for experimental error.

Table 5

T	*E*	[a] k_p	[b] $\hat{k}_p$	Coefficients from the least-squares regression	t	[c] Adjusted Model	
-1	-1	0.0043	0.0061	0.0103	30.29	0.0103	*i.t.*
1	-1	0.0142	0.0148	0.0043	16.07	0.0043	*T*
-1	1	0.0039	0.0061	0.0003	1.24	NS	*E*
1	1	0.0151	0.0148	0.0003	0.83	NS	*TE*
-1.41	0	0.0098	0.0074	0.0016	5.60	0.0016	T^2
1.41	0	0.0195	0.0197	-0.0015	5.15	-0.0015	E^2
0	-1.41	0.0076	0.0074				
0	1.41	0.0092	0.0074				
0	0	0.0096	0.0103	Average value	= 0.0104		
0	0	0.0108	0.0103	Expected average value	= 0.0103		
0	0	0.0097	0.0103	Var(Ee)	< 0.00001		
0	0	0.0101	0.0103	t(α<0.05; υ=4)	= 2.776		
0	0	0.0114	0.0103				

	[d] SS	[e] υ	[f] MS	[g] Mean Squares Ratios	
Model	0.00019	3	0.000063	MSM/MSE= 28.34	F_9^3 (α=0.05)= 3.863
Error	0.00002	9	0.000002	MSMLF/MSM= 0.410	F_3^8 (α=0.05)= 8.845
Exp. Error	0.000002	4	0.000001	MSE/MSEe= 3.818	F_4^9 (α=0.05)= 5.999
Lack of fitting	0.00002	5	0.000004	MSLF/MSEe= 6.073	F_4^5 (α=0.05)= 6.256
Total	0.00021	12			

r^2= 0.904

r^2 adjusted= 0.872

[a]Experimental values of the specific rates of total phenolic production. [b]Estimated values of the specific rates of total phenolic production from the adjusted model. [c]Coefficients for the terms of the adjusted model: i.t., independent term; E, ethanol concentration (%); T, temperature (ºC); NS, not significant coefficient. [d]SS: sum of squares. [e]υ: degrees of freedom. [f]MS: mean squares. [g]Mean Square Ratios: MSM, mean squares of the model; MSE, mean squares for error; MSMLF, mean squares for model lack of fit; MSEe, mean squares for experimental error.

Table 6

T	*E*	[a] D_m	[b] $\hat{D}_m$	Coefficients from the least-squares regression	t	[c] Adjusted Model	
-1	-1	65.51	66.13	80.45	169.31	80.45	*i.t.*
1	-1	74.48	72.82	3.35	8.90	3.35	*T*
-1	1	61.32	66.13	-0.21	0.55	NS	*E*
1	1	70.71	72.82	0.10	0.20	NS	*TE*
-1.41	0	70.32	67.03	-4.35	10.76	-4.35	T^2
1.41	0	76.23	76.52	-6.62	16.37	-6.62	E^2
0	-1.41	66.53	67.28				
0	1.41	70.99	67.28				
0	0	79.34	80.45	Average value	= 73.716		
0	0	79.91	80.45	Expected average value	= 80.443		
0	0	82.02	80.45	Var(Ee)	= 1.1290		
0	0	79.96	80.45	t(α<0.05; υ=4)	= 2.776		
0	0	80.98	80.45				

	[d] SS	[e] υ	[f] MS	[g] Mean Squares Ratios	
Model	478.29	3	159.430	MSM/MSE= 23.88	F_9^3 (α=0.05)= 3.863
Error	60.09	9	6.677	MSMLF/MSM= 0.419	F_3^8 (α=0.05)= 8.845
Exp. Error	4.52	4	1.129	MSE/MSEe= 5.914	F_4^9 (α=0.05)= 5.999
Lack of fitting	55.58	5	11.115	-	-
Total	538.38	12			

r^2=	0.881
r^2 adjusted=	0.851

[a]Experimental values of maximum DPPH scavenging activity. [b]Estimated values of maximum DPPH scavenging activity from the adjusted model. [c]Coefficients for the terms of the adjusted model: i.t., independent term; E, ethanol concentration (%); T, temperature (ºC); NS, not significant coefficient. [d]SS: sum of squares. [e]υ: degrees of freedom. [f]MS: mean squares. [g]Mean Square Ratios: MSM, mean squares of the model; MSE, mean squares for error; MSMLF, mean squares for model lack of fit; MSEe, mean squares for experimental error.

Table 7

T	*E*	[a] k_d	[b] $\hat{k}_d$	Coefficients from the least-squares regression	t	[c] Adjusted Model	
-1	-1	0.0152	0.0164	0.0198	47.28	0.0198	*i.t.*
1	-1	0.0248	0.0258	0.0047	14.22	0.0047	*T*
-1	1	0.0232	0.0203	0.0019	5.86	0.0019	*E*
1	1	0.0284	0.0297	-0.0011	2.32	NS	*TE*
-1.41	0	0.0147	0.0160	0.0015	4.09	0.0015	T^2
1.41	0	0.0308	0.0293	0.0018	5.12	0.0018	E^2
0	-1.41	0.0221	0.0206				
0	1.41	0.0249	0.0261				
0	0	0.0199	0.0198	Average value	= 0.0218		
0	0	0.0206	0.0198	Expected average value	= 0.0198		
0	0	0.0192	0.0198	Var(Ee)	< 0.00001		
0	0	0.0185	0.0198	t(α<0.05; υ=4)	= 2.776		
0	0	0.0206	0.0198				

	[d] SS	[e] υ	[f] MS	[g] Mean Squares Ratios	
Model	0.00024	4	0.000060	MSM/MSE= 20.52	F_8^4 (α=0.05)= 3.838
Error	0.00002	8	0.000003	MSMLF/MSM= 0.541	F_4^8 (α=0.05)= 6.041
Exp. Error	0.000003	4	0.000001	MSE/MSEe= 3.349	F_4^8 (α=0.05)= 6.041
Lack of fitting	0.00002	4	0.000005	MSLF/MSEe= 5.698	F_4^4 (α=0.05)= 6.388
Total	0.00026	12			

r^2=	0.911
r^2 adjusted=	0.867

[a]Experimental values of the specific rates of DPPH scavenging activity. [b]Estimated values of the specific rates of DPPH scavenging activity from the adjusted model. [c]Coefficients for the terms of the adjusted model: i.t., independent term; E, ethanol concentration (%); T, temperature (ºC); NS, not significant coefficient. [d]SS: sum of squares. [e]υ: degrees of freedom. [f]MS: mean squares. [g]Mean Square Ratios: MSM, mean squares of the model; MSE, mean squares for error; MSMLF, mean squares for model lack of fit; MSEe, mean squares for experimental error.

FIGURE 1

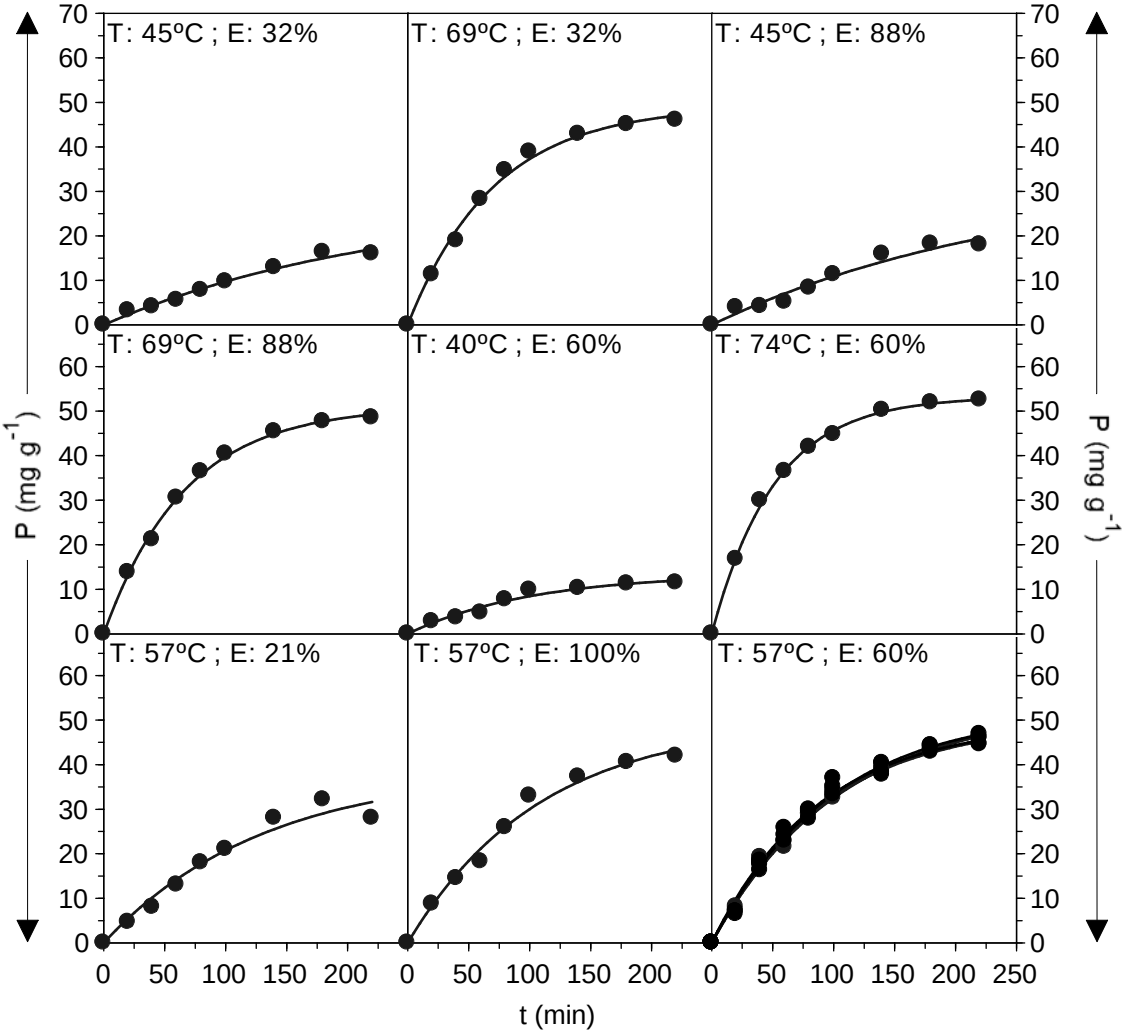
T: 45ºC ; E: 32%
T: 69ºC ; E: 32%
T: 45ºC ; E: 88%
T: 69ºC ; E: 88%
T: 40ºC ; E: 60%
T: 74ºC ; E: 60%
T: 57ºC ; E: 21%
T: 57ºC ; E: 100%
T: 57ºC ; E: 60%
P (mg g-1)
t (min)

FIGURE 2

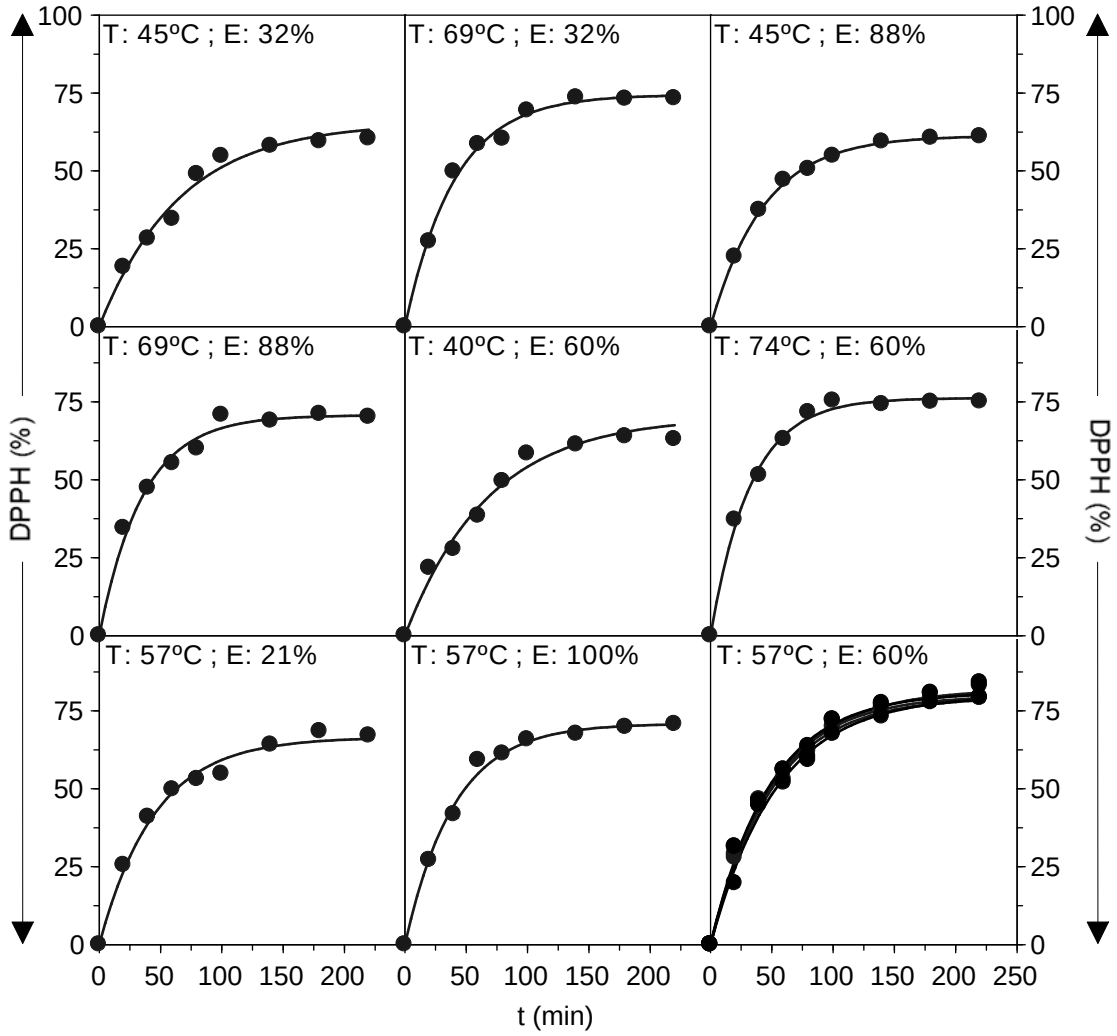
T: 45ºC ; E: 32%
T: 69ºC ; E: 32%
T: 45ºC ; E: 88%
T: 69ºC ; E: 88%
T: 40ºC ; E: 60%
T: 74ºC ; E: 60%
T: 57ºC ; E: 21%
T: 57ºC ; E: 100%
T: 57ºC ; E: 60%
DPPH (%)
DPPH (%)
t (min)
0
25
50
75
100
0
50
100
150
200
250

FIGURE 3

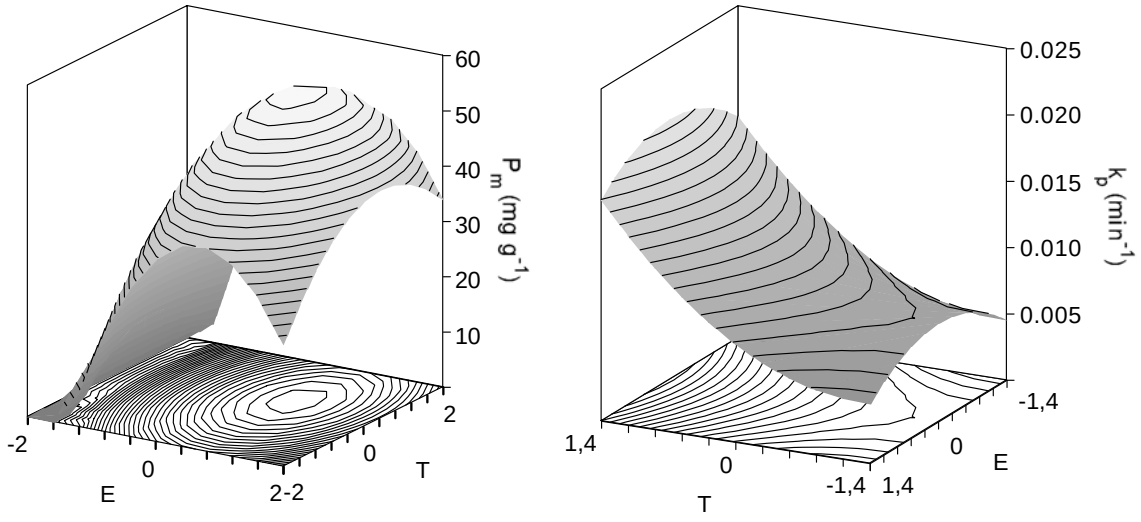
60
50
40
30
20
10
P_m (mg g^{-1})
-2
0
2-2
E
0
2
T
0.025
0.020
0.015
0.010
0.005
k_p (min^{-1})
1,4
0
-1,4 1,4
T
0
-1,4
E

FIGURE 4