Optimization of antioxidants extraction from soybeans fermented by
Aspergillus oryzae

Dyah H. Wardhani¹, José A. Vázquez^{1,2} and Severino S. Pandiella^{1*}

¹ School of Chemical Engineering and Analytical Science
The University of Manchester
Sackville Street, PO Box 88, Manchester M60 1QD, UK

² Grupo de Reciclado y Valorización de Materiales Residuales
Instituto de Investigaciones Mariñas (CSIC)
r/ Eduardo Cabello, 6. Vigo-36208. Galicia – Spain

* Corresponding author e-mail: s.pandiella@manchester.ac.uk

Tel +44 (0)161 306 4429, Fax +44 (0) 161 306 9321

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°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°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 (Naczka, & Shahidi, 2006; Calliste, Trouillas, Allais, Simon, & Duroux, 2001). Limited information is

available regarding the extraction of antioxidant compounds in fermented soybeans. However, significant higher concentration of phenolics was obtained after fermentation when compare to unfermented soybeans (McCue, & Shetty, 2003; Esaki, Onozaki, Kawakishi, & Osawa, 1997; Lin, Wei, & Chou, 2006; Wardhani, Vázquez, & Pandiella, 2009). A universal extraction protocol would be difficult to establish due to the complex composition of the beans and the structural diversity of the antioxidant compounds of the natural source. The extraction efficiency is affected by multiple variables, amongst which temperature and the nature of the solvent are the most important factors, which may act dependently or independently (Liu, & Ang, 2000).

Processes are commonly optimised using one-factor-at-a-time approaches. Optimal conditions or interactions between variables cannot be predicted with this methodology. This limitation can be overcome using experimental design methodologies (DOE; Box, Hunter, & Hunter, 1989; Akhnazarova, & Kafarov, 1982). DOE is a collection of statistical and mathematical techniques that have been successfully used in developing, improving and optimizing bio-processes (Liyana-Pathirana, & Shahidi, 2005; Juntachote, Berghofer, Bauer, & Siebenhandl, 2006; Paz, Vázquez, Riobó, & Franco, 2006; Vázquez, González, & Murado, 2006; Bandeira, Tininis, Bolzani, & Cavaleiro, 2006).

In this study, the optimal conditions for antioxidant extraction from soybeans fermented with *Aspergillus oryzae* were investigated using two complementary and sequential approaches. A factorial design was initially proposed. Kinetic analyses were then performed at the temperature-ethanol concentration points of the design. The parameters 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.

MATERIALS 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°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°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°C for 5 days. Soybean samples were crushed with mortar and pestle before sealed in plastic bag and store at -30°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

extract was centrifugated at $16,249 \times g$ for 5 min, and the supernatant was used for the antioxidant determination.

Determination of total phenolic content

The total phenolic content was determined based on the method of Singleton, Orthofer, and Lamuela-Raventós (1999), using the Folin-Ciocalteu Reagent (FCR) with gallic acid as a standard. 50 μ L of sample or blank were added to 3 mL of distilled water in 12 mL test tubes. A volume of FCR (250 μ L) was placed into the tube and mixed before adding 750 μ L of saturated Na_2CO_3 . The final volume of the reaction mixture was adjusted to 5 mL with distilled water. The absorbance at 765 nm was read in 1-cm cuvettes after incubation for 2 h at room temperature, and readings were compared with a standard curve of gallic acid. The total phenolic content was expressed as mg of gallic acid equivalent per gram dry basis of fermented soybeans (mg GAE /g db).

Determination of DPPH radical scavenging activity

The effect of the extract on 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) radical was estimated according to the procedure described by Brand-Williams, Cuvelier, and Berset (1995). The extract (0.1 mL) was added to 3.9 mL of DPPH 6×10^{-5} M in methanol which was prepared daily. The decrease in absorbance was determined at 515 nm after incubation for 30 min. A DPPH solution without sample was used as control and the DPPH percentage inhibition was calculated according to the following equation:

$$\text{DPPH scavenging effect (\%)} = \left(1 - \frac{\text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}} \right) \times 100\% \quad (1)$$

Experimental design and statistical analysis

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

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

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

160

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

161

162 **RESULTS AND DISCUSSION**

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

170

171 **Kinetics of antioxidants activities**

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

177

$$178 \quad P = P_m \cdot \left(1 - e^{-k_p \cdot t}\right) \quad (2)$$

179

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

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

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 (74°C). 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 polynomial function

$$R=b_0+ b_1 T + b_2 E + b_{12} TE + b_{11} T^2 + b_{22} E^2 \quad (4)$$

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 extraction; $T_m = 0.690$ and $E_m = 0.467$ in codified values, equivalent to 65.3°C 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

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

The maximum D_m was found at $T_m = 0.385$ (61.6°C) and $E_m = 0$ (60%). At this point the predicted maximum DPPH scavenging activity was 81.6%.

Table 7 summarises the results of the factorial design for the specific rate of DPPH scavenging activity (k_d) plotted in Figure 4 (right). Only the combined term TE was not significant, and the model defines a concave response surface with a line of maximum slope in the proximity of $E=0$. As for the k_p model, a maximum response cannot be calculated within the experimental domain, but k_d increases with the temperature.

The empirical models obtained show a good fitting and consistency. The correlation with the observed values (r^2_{adjusted}) was higher than 0.85 and the experimental variability of the replica in the centre of the experimental domain was considerably low, allowing for construction of highly predictive models.

The improvement of the antioxidant extraction with temperature was probably due to the increasing diffusivity of the solvent in the solid matrix and the solubility of the phenolic compounds in the solvent, which favour the extraction (Juntachote, Berghofer, Bauer, & Siebenhandl, 2006; Cacace, & Mazza, 2003; Herrero, Martin-Alvarez, Señoráns, Cifuentes, & Ibáñez, 2005). However, it should be noted that increasing temperature beyond a certain value can lead to decomposition of some phenolic compounds. Rostagno, Palma, and Barroso (2007) reported decomposition of isoflavones in soybean during heat treatments. Malonyl isoflavones also degrade when extraction is performed between 75 and 100°C. Extraction between 100-125°C affects acetyl isoflavones and higher temperatures sharply reduced the glucosides concentrations.

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

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

CONCLUSIONS

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

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

ACKNOWLEDGEMENTS

The authors wish to acknowledge the SPMU-TPSD Diponegoro University (Indonesia) for the grant awarded to Dyah Hesti Wardhani to do this research (ADB loan N° 1792-INO). Drs. Pablo Fuciños and José Antonio Vázquez has been awarded a postdoctoral grant (Programa de bolsas para estadias fóra de Galicia, 2007 and 2008 respectively) by the Dirección Xeral de Investigación, Desenvolvemento e Innovación, Xunta de Galicia, Spain.

REFERENCES

- Akhnazarova, S., & Kafarov, V. (1982). *Experiment optimization in chemistry and chemical engineering*. Moscow: MIR Publishers.
- Bandeira, K. F., Tininis, A. G, Bolzani, V. D. S, & Cavaleiro, A. J. (2006). Optimisation of conditions for the extraction of casearins from *Caesaria sylvestris* using response surface methodology. *Phytochemical Analysis*, 17, 168-175.

Box, G. E., Hunter, J. S., & Hunter, W. G. (2005). Statistics for experimenters: design, innovation, and discovery. John Wiley & Sons, Inc., Hoboken, New Jersey.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und Technologie*, 28, 25-30.

Cacace, J. E., & Mazza, G. (2003). Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering*, 59, 379-389.

Calliste, C. A., Trouillas, P., Allais, D. P., Simon, A., & Duroux, J. L. (2001). Free radical scavenging activities measured by electron spin resonance spectroscopy and B16 cell antiproliferative behaviors of seven plants. *Journal of Agricultural and Food Chemistry*, 49, 3321-3327.

Cuppett, S., Schnepf, M., & Hall, C. (1997). Natural antioxidants-are they a reality? In: F. Shahidi, *Natural antioxidants: chemistry, health effects, and applications* (pp. 12-24). Illinois: AOCS Press, Illinois.

Da Silva Pinto, M., Lajolo, F. M., & Genovese, M. I. (2005). Effect of storage temperature and water activity on the content and profile of isoflavones, antioxidant activity, and in vitro protein digestibility of soy protein isolates and defatted soy flours. *Journal of Agricultural and Food Chemistry*, 53, 6340-6346.

342 Esaki, H., Onozaki, H., Kawakishi, S., & Osawa, T. (1997). Antioxidant activity and
 343 isolation from soybeans fermented with *Aspergillus* spp. *Journal of Agricultural and Food*
 344 *Chemistry*, 45, 2020-2024.

345

346 Halliwell, B., & Gutteridge, J. M. C. (1999). *Free radicals in Biology and Medicine*, 3rd
 347 *edition*. Oxford: Oxford University Press.

348

349 Hayes, R. E., Bookwalter, G. N., & Bagley, E.B. (1977). Antioxidant activity of soybean
 350 flour and derivatives — a review. *Journal of Food Science*, 42, 1527-1532.

351

352 Herrero, M., Martin-Alvarez, P. J., Señoráns, F. J., Cifuentes, A., & Ibáñez, E. (2005).
 353 Optimization of accelerated solvent extraction of antioxidants from *Spirulina plantesis*
 354 microalga. *Food Chemistry*, 93, 417-423.

355

356 Hinneburg, I., & Neubert, R. H. H. (2005). Influence of extraction parameters of the
 357 phytochemical characteristics of extracts from buckwheat (*Fagopyrum esculentum*) herb.
 358 *Journal of Agricultural and Food Chemistry*, 53, 3-7.

359

360 Hubert, J., Berger, M., Nepveu, F., Paul, F., & Daydé, J. (2008). Effects of fermentation on
 361 the phytochemical composition and antioxidant properties of soy germ. *Food Chemistry*,
 362 109, 709-721.

363

364 Isanga, J. & Zhang, G.-N. (2008). Soybean bioactive components and their implications to
 365 health - A review. *Food Reviews International*, 24, 252-276.

366

367 Juntachote, T., Berghofer, E., Bauer, F., & Siebenhandl, S. (2006). The application of
368 response surface methodology to the production of phenolic extracts of lemon grass,
369 galangal, holy basil and rosemary. *International Journal of Food Science & Technology*, 41,
370 121-133.

371

372 Lin, C. H., Wei, Y. T., & Chou, C. C. (2006). Enhanced antioxidative activity of soybean
373 koji prepared with various filamentous fungi. *Food Microbiology*, 23, 628-633.

374

375 Liu, F. F., Ang, C. Y. W., & Springer, D. (2000). Optimization of extraction conditions for
376 active components in *Hypericum perforatum* using response surface methodology. *Journal*
377 *of Agricultural and Food Chemistry*, 48, 3364-3371.

378

379 Liyana-Pathirana, C., & Shahidi, F. (2005). Optimization of extraction of phenolic
380 compounds from wheat using response surface methodology. *Food Chemistry*, 93, 47-56.

381

382 McCue, P., & Shetty, K. (2003). Role of carbohydrate-claving enzyme in phenolic
383 antioxidant mobilization from whole soybean fermented with *Rhizopus oligosporus*. *Food*
384 *Biotechnology*, 17, 27-37.

385

386 Naczk, M., & Shahidi, F. (2006). Phenolics in cereal, fruits and vegetables: Occurrence,
387 extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis*, 41, 1523-
388 1542.

389

390 Paz, B., Vázquez, J. A., Riobó, P., & Franco, J. M. (2006). Study of the effect of
 391 temperature, irradiance and salinity on growth and yessotoxin production by the
 392 dinoflagellate *Protoceratium reticulatum* in culture by using a kinetic and factorial
 393 approach. *Marine Environmental Research*, 62, 286-300.
 394
 395 Pokorny, J. (2003). Natural antioxidants. In: P. Zeuthen, & L. S. Sorensen, *Food*
 396 *preservation techniques* (pp. 31-48). Cambridge: Woodhead Publishing Ltd.
 397
 398 Pratt, D. E., & Birac, P. M. (1979). Source of antioxidant activity of soybeans and soy
 399 products. *Journal of Food Science*, 44, 170-1722.
 400
 401 Pratt, D. E., & Hudson, B. J. F. (1990). Natural antioxidants not exploited commercially. In:
 402 B. J. F. Hudson, *Food antioxidants* (pp. 171-191). London: Elsevier Science Publishers Ltd.
 403
 404 Romero, A. M., Doval, M. M., Sturla, M. A., & Judis, M. A. (2004). Antioxidant properties
 405 of polyphenol-containing extract from soybean fermented with *Saccharomyces cerevisiae*.
 406 *European Journal of Lipid Science and Technology*, 106, 424-431.
 407
 408 Rostagno, M. A., Palma, M., & Barroso, C. G. (2007). Microwave assisted extraction of
 409 soy isoflavones. *Analytica Chimica Acta*, 588, 274-282.
 410
 411 Shahidi, F., & Wanasundara, U. N. (1997). Measurement of lipid oxidation and evaluation
 412 of antioxidant activity. In: F. Shahidi, *Natural antioxidants: chemistry, health effects, and*
 413 *applications* (pp. 379-396). Illinois: AOCS Press.

414

415 Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total
416 phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu
417 reagent. In: L. Packer, *Methods in Enzimology*, vol. 299 (pp. 152-178), Oxidants and
418 antioxidants. San Diego CA: Academic Press.

419

420 Tsao, R., & Deng, Z. (2004). Separation procedures for naturally occurring antioxidant
421 phytochemicals. *Journal of Chromatography B*, 812, 85-99.

422

423 Vázquez, J. A., González, M. P., & Murado, M. A. (2006). Preliminary assays on the nisin
424 and pediocin production using waste protein sources. Factorial and kinetic studies.
425 *Bioresource Technology*, 97, 605-613.

426

427 Wardhani, D. H., Vázquez, J. A., & Pandiella, S. S. (2008). Kinetics of daidzin and genistin
428 transformations and water absorption during soybean soaking at different temperatures.
429 *Food Chemistry*, 111, 13-19.

430

431 Wardhani, D. H., Vázquez, J. A., & Pandiella, S. S. (2009). Mathematical modeling of the
432 development of antioxidant activity in soybeans fermented with *Aspergillus oryzae* and
433 *Aspergillus awamori* in the solid state. *Journal of Agricultural and Food Chemistry*, 57,
434 540-544.

435

436 Wettasinghe, M., & Shahidi, F. (1999). Antioxidant and free radical-scavenging properties
437 of ethanolic extracts of defatted borage (*Borago officinalis* L.) seeds. *Food Chemistry*, 67,
438 399-414.
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.

TABLE CAPTIONS

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

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

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

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

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

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

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

^aMaximum total phenolic concentration. ^bConfidence intervals ($\alpha = 0.05$; $df = 7$). ^cSpecific rates of total phenolic production.

^dF-Fisher test (df_1 = degrees of freedom of the model; df_2 = degrees of freedom of the error)

^eCorrelation 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	r (O:P)
T : -1 (45°C); E : -1 (32%)	65.51 $\pm$ 6.11	0.0152 $\pm$ 0.0041	1124.24	<0.0001	0.992
T : 1 (69°C); E : -1 (32%)	74.48 $\pm$ 3.17	0.0248 $\pm$ 0.0037	3386.46	<0.0001	0.997
T : -1 (45°C); E : 1 (88%)	61.32 $\pm$ 1.03	0.0232 $\pm$ 0.0013	23578.91	<0.0001	0.999
T : 1 (69°C); E : 1 (88%)	70.71 $\pm$ 3.75	0.0284 $\pm$ 0.0057	1978.03	<0.0001	0.994
T : -1.41 (40°C); E : 0 (60%)	70.32 $\pm$ 6.65	0.0147 $\pm$ 0.0042	1071.03	<0.0001	0.991
T : 1.41 (74°C); E : 0 (60%)	76.23 $\pm$ 2.49	0.0308 $\pm$ 0.0041	4860.86	<0.0001	0.998
T : 0 (57°C); E : -1.41 (21%)	66.53 $\pm$ 4.03	0.0221 $\pm$ 0.0042	1961.18	<0.0001	0.995
T : 0 (57°C); E : 1.41 (100%)	70.99 $\pm$ 3.02	0.0249 $\pm$ 0.0037	3453.07	<0.0001	0.997
T : 0 (57°C); E : 0 (60%)	79.34 $\pm$ 2.76	0.0199 $\pm$ 0.0021	6576.57	<0.0001	0.999
T : 0 (57°C); E : 0 (60%)	79.91 $\pm$ 2.14	0.0206 $\pm$ 0.0017	10498.72	<0.0001	0.999
T : 0 (57°C); E : 0 (60%)	82.02 $\pm$ 6.11	0.0192 $\pm$ 0.0039	1569.18	<0.0001	0.994
T : 0 (57°C); E : 0 (60%)	79.96 $\pm$ 5.15	0.0185 $\pm$ 0.0034	2103.97	<0.0001	0.996
T : 0 (57°C); E : 0 (60%)	80.98 $\pm$ 3.70	0.0206 $\pm$ 0.0026	4099.41	<0.0001	0.997

^aMaximum DPPH scavenging activity. ^bConfidence intervals ($\alpha = 0.05$; $df = 7$). ^cSpecific rates of DPPH scavenging activity.

^dF-Fisher test (df_1 = degrees of freedom of the model; df_2 = degrees of freedom of the error)

^eCorrelation coefficient between observed and predicted data.

542 **Table 4**
543

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>i.t.</i>
1	-1	49.15	48.57	11.92	20.16	11.92	<i>T</i>
-1	1	33.59	30.61	2.94	4.97	2.94	<i>E</i>
1	1	51.08	54.44	-1.01	1.21	NS	<i>TE</i>
-1.41	0	13.54	17.38	-8.66	13.62	-8.66	<i>T²</i>
1.41	0	53.26	50.98	-3.15	4.95	-3.15	<i>E²</i>
0	-1.41	38.86	40.99				
0	1.41	49.87	49.28				
0	0	52.85	51.39				
0	0	49.80	51.39				
0	0	52.55	51.39				
0	0	52.41	51.39				
0	0	49.37	51.39				
				Average value = 44.149			
				Expected average value = 51.396			
				Var(Ee) = 2.7867			
				t($\alpha < 0.05$; $\nu = 4$) = 2.776			
		d SS	$^e \nu$	f MS	g Mean Squares Ratios		
Model		1747.66	4	436.92	MSM/MSE = 53.99	F_8^4 ($\alpha = 0.05$) = 3.838	
Error		64.74	8	8.092	MSMLF/MSM = 0.515	F_4^8 ($\alpha = 0.05$) = 6.041	
Exp. Error		11.15	4	2.787	MSE/MSEe = 2.904	F_4^8 ($\alpha = 0.05$) = 6.041	
Lack of fitting		53.59	4	13.398	MSLF/MSEe = 4.808	F_4^4 ($\alpha = 0.05$) = 6.388	
Total		1812.40	12				
					$r^2 =$	0.964	
					r^2 adjusted =	0.946	

544 a Experimental values of maximum total phenolic concentration. b Estimated values of maximum total phenolic concentration from the
545 adjusted model. c Coefficients for the terms of the adjusted model: i.t., independent term; E, ethanol concentration (%); T, temperature
546 ($^{\circ}$ C); NS, not significant coefficient. d SS: sum of squares. $^e \nu$: degrees of freedom. f MS: mean squares. g Mean Square Ratios: MSM,
547 mean squares of the model; MSE, mean squares for error; MSMLF, mean squares for model lack of fit; MSEe, mean squares for
548 experimental error.

Table 5

T	E	$^a k_p$	$^b \hat{k}_p$	Coefficients from the least-squares regression	t	Adjusted Model	
-1	-1	0.0043	0.0061	0.0103	30.29	0.0103	<i>i.t.</i>
1	-1	0.0142	0.0148	0.0043	16.07	0.0043	<i>T</i>
-1	1	0.0039	0.0061	0.0003	1.24	NS	<i>E</i>
1	1	0.0151	0.0148	0.0003	0.83	NS	<i>TE</i>
-1.41	0	0.0098	0.0074	0.0016	5.60	0.0016	<i>T²</i>
1.41	0	0.0195	0.0197	-0.0015	5.15	-0.0015	<i>E²</i>
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; ν=4) = 2.776			
0	0	0.0114	0.0103				
		dSS	eν	fMS	gMean 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

^aExperimental values of the specific rates of total phenolic production. ^bEstimated values of the specific rates of total phenolic production from the adjusted model. ^cCoefficients for the terms of the adjusted model: i.t., independent term; E, ethanol concentration (%); T, temperature (°C); NS, not significant coefficient. ^dSS: sum of squares. ^e ν : degrees of freedom. ^fMS: mean squares. ^gMean 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>i.t.</i>
1	-1	74.48	72.82	3.35	8.90	3.35 <i>T</i>
-1	1	61.32	66.13	-0.21	0.55	NS <i>E</i>
1	1	70.71	72.82	0.10	0.20	NS <i>TE</i>
-1.41	0	70.32	67.03	-4.35	10.76	-4.35 <i>T²</i>
1.41	0	76.23	76.52	-6.62	16.37	-6.62 <i>E²</i>
0	-1.41	66.53	67.28	Average value = 73.716 Expected average value = 80.443 Var(Ee) = 1.1290 t($\alpha < 0.05$; $\nu = 4$) = 2.776		
0	1.41	70.99	67.28			
0	0	79.34	80.45			
0	0	79.91	80.45			
0	0	82.02	80.45			
0	0	79.96	80.45			
0	0	80.98	80.45			
	d SS	$^e \nu$	f MS	g Mean Squares Ratios		
Model	478.29	3	159.430	MSM/MSE= 23.88	$F_9^3 (\alpha=0.05)= 3.863$	
Error	60.09	9	6.677	MSMLF/MSM= 0.419	$F_3^8 (\alpha=0.05)= 8.845$	
Exp. Error	4.52	4	1.129	MSE/MSEe= 5.914	$F_4^9 (\alpha=0.05)= 5.999$	
Lack of fitting	55.58	5	11.115	-	-	
Total	538.38	12				
				$r^2=$	0.881	
				r^2 adjusted=	0.851	

^aExperimental values of maximum DPPH scavenging activity. ^bEstimated values of maximum DPPH scavenging activity from the adjusted model. ^cCoefficients for the terms of the adjusted model: i.t., independent term; E, ethanol concentration (%); T, temperature (°C); NS, not significant coefficient. ^dSS: sum of squares. ^e ν : degrees of freedom. ^fMS: mean squares. ^gMean 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	Adjusted Model	
-1	-1	0.0152	0.0164	0.0198	47.28	0.0198	<i>i.t.</i>
1	-1	0.0248	0.0258	0.0047	14.22	0.0047	<i>T</i>
-1	1	0.0232	0.0203	0.0019	5.86	0.0019	<i>E</i>
1	1	0.0284	0.0297	-0.0011	2.32	NS	<i>TE</i>
-1.41	0	0.0147	0.0160	0.0015	4.09	0.0015	<i>T²</i>
1.41	0	0.0308	0.0293	0.0018	5.12	0.0018	<i>E²</i>
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; ν=4) = 2.776			
0	0	0.0206	0.0198				
		dSS	eν	fMS	gMean 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

^aExperimental values of the specific rates of DPPH scavenging activity. ^bEstimated values of the specific rates of DPPH scavenging activity from the adjusted model. ^cCoefficients for the terms of the adjusted model: i.t., independent term; E, ethanol concentration (%); T, temperature (°C); NS, not significant coefficient. ^dSS: sum of squares. ^e ν : degrees of freedom. ^fMS: mean squares. ^gMean 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

FIGURE 2

FIGURE 3

FIGURE 4