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Kinetics of Thermal Modifications in a Grape Seed Extract

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Supporting Information

ABSTRACT: The thermal modification kinetics of a commercial grape seed extract (GSE) was investigated. A GSE was exposed to 60, 90, and 120 °C for 5, 10, 15, 30, 45, and 60 min. The antioxidant activity (AA) and the absorbance at 420 nm (A_{420}) were measured. (+)-Catechin, (−)-epicatechin, procyanidins B1 and B2, and gallic acid were identified and measured. After the thermal treatments, the AA did not show a significant difference ($p > 0.05$) and both procyanidins and gallic acid increased as well as A_{420}. (+)-Catechin and (−)-epicatechin decreased. To obtain the activation energy (E_a) of the changes, a modified Weibull and a combined zero- and first-order model were compared, both followed by the Arrhenius equation. The Weibull model was more accurate. The E_a values for browning and (+)-catechin, (−)-epicatechin, gallic acid, and procyanidins B1 and B2 were 170, 286, 42, 102, 249, and 95 kJ/mol, respectively. The results were valid at a confident level of 95%.

KEYWORDS: Polyphenols, grape seed, kinetics, thermal treatments, flavan-3-ols

INTRODUCTION

It is known that polyphenols have a beneficial effect on human health; their free-radical scavenging property protects cells from lesions caused by these highly reactive molecules. Other beneficial effects from polyphenols on human health are anti-inflammatory and anti-allergenic properties; polyphenols can also reduce the risk of cardiovascular diseases and have potential cancer chemopreventive activities[1]

Grape seeds are a rich source of polyphenol compounds, especially phenolic acids, flavan-3-ols, such as catechins and their isomers, and proanthocyanidins.[2] Therefore, grape seed extracts (GSEs) could be a suitable functional ingredient to be employed in a wide variety of food products. Functional foods help to compensate imbalanced diets and make up for food disorders because they exert a preventive role, reducing the risk of diseases. However, the functional food and nutraceutical industry is increasingly facing challenges in incorporating bioactive and health-promoting ingredients into food without compromising their biofunctionality.[3] One factor that can affect the composition and biofunctionality of nutraceutical ingredients is heat applied to these ingredients during food elaboration processes.

With regard to the thermal stability of flavan-3-ols, many studies have been made on tea extracts in which the concentration of catechins, such as (−)-epigallocatechin gallate (EGCG), (−)-epicatechin (EPI), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin (EGC), decreased when the samples were heat-treated at a temperature range of 70−120 °C for a wide variety of times.[4,5] Visual changes also occurred when tea extract was heated; an increase in the optical density at 390 nm was observed after pasteurizing green and black teas.[6] The stability of polyphenols from other sources have also been studied; Pacheco-Palencia et al.[7] found that, after heating açai oil at 170 °C for 20 min, its phenolic content decreased 10%. Heating red grape marcs at 200 °C for 30 min reduces its antioxidant capacity by 15% and its phenolic content by 30%.[8] Pasteurization and storage of fruit juices also reduce its phenolic content. When apple juice was stored at 80 °C for 100 h, its EPI concentration was reduced 80%.[9] Increases in the phenolic content after stem-cooking vegetables have also been reported, which may be due to an enhanced availability for their extraction.[10]

In studies made specifically on GSE, it was found that thermal processing of bread caused a decrease in the antioxidant activity of an added GSE by around 30−40%.[11] GSE kept its antioxidant properties after being added to pork meat and heated at 180 °C for at least 8 min.[12] Kim et al.[13] found that submitting grape seeds to 150 °C for 40 min increased its phenolic content by 50%, but raising the temperature to 200 °C reduced its phenolic content and antioxidant activity.

One way to quantify and predict changes in the phenolic content and antioxidant activity of the extracts, after being submitted to thermal conditions, is using mathematical models. The phenolic composition of a green tea extract showed that epimerization and degradation of tea catechins followed first-order reactions and the rate constants of reaction kinetics followed the Arrhenius equation.[14] The same reactions were found when tea extracts were added to bread, showing that epimerization and degradation of catechins is faster in the crust than in the crumb.[14] In the study made by Cisse et al.,[15] the impact of the temperature (30−90 °C) on anthocyanins in blood orange juice, two tropical highland blackberry juices, and four roselle extracts was evaluated and the data showed that thermal degradation of anthocyanins can be described using first-order reaction kinetics.

Considering the susceptibility of GSE to heat, the aim of this study is to evaluate the stability to thermal processing of a commercial polyphenolic extract from grape seeds by creating mathematical models to analyze and quantify the changes in their main individual phenolic compounds, as well as changes in their antioxidant activity and browning.

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MATERIALS AND METHODS

Materials. A commercial GSE provided by Exxentia (Spain) obtained from grape seeds by an extraction with a hydroalcoholic solution was used in the study. Methanol (HPLC grade), ethanol (absolute), Folin−Ciocalteu reagent, 1-butanol, hydrochloric acid (37%), perchloric acid (60%), sodium carbonate, ferric sulfate heptahydrate, potassium peroxodisulfate, and gallic acid (GA) were purchased from Panreac (Spain). Cyanidin-3-*O*-glucoside was purchased from Polyphenols (Norway). 2,2-Anzino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and (+)-catechin (CAT) were purchased from Sigma Chemical Co. (Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical (Germany). EPI was purchased from Fluka (Germany). Procyanidin B1 (ProB1) and procyanidin B2 (ProB2) were purchased from Extrasynthese (France).

Thermal Treatments. The GSE was dissolved in a hydroalcoholic solution (4%, v/v) at a concentration of 2.5 g/L, which is the highest achievable concentration to obtain a stable solution using the minimum possible amount of ethanol. The GSE solution was put in hermetic closed glass bottles to be submitted to 60, 90, and 120 °C for 5, 10, 15, 30, 45, and 60 min using a water bath pressure autoclave HJ Marrodan L1581 (Navarre, Spain); the treatments were made by duplicate and two repetitions per replica. The temperature and time ranges were selected to simulate the conditions commonly used in the food industry. After the treatments, the samples were stored at 4 °C without oxygen for 1−2 days prior to analysis. Before the essays, the samples were centrifuged at 10.7×10^3 g for 5 min in a centrifuge Sigma 3K30 (GMBH, Germany). To measure the phenolic and tannin content as well as the browning, the samples were diluted 5 times, and to measure the antioxidant activity, the samples were diluted 25 times.

Humidity and pH. The humidity of the GSE was measured in a moisture balance ST-H50 (Gram Precision, Spain) at 105 °C, taking measures every 20 s until three were equal and using 0.5 g of GSE every time. The humidity was expressed as a percentage.

The pH values were measured using a pH-meter model Crison Basic 20 (Alella, Spain) fitted with a glass electrode.

Total Phenolic Content (TPC). The Folin−Ciocalteu method was employed[16] to obtain the TPC. In a 100 mL volumetric flask, 1 mL of the diluted extract, 50 mL of deionized water, 5 mL of the Folin−Ciocalteu reagent, and 20 mL of a 20% (w/v) sodium carbonate solution were added, in that order. The volumetric flask was filled to its volume with deionized water. After 30 min, the absorbance of the samples was measured at 750 nm in a Cintra 20 (GMBH, Germany) double-beam spectrophotometer. The phenolic content was expressed in gallic acid equivalents after the preparation of a standard curve of gallic acid from 0 to 600 mg/L ($r^2 = 0.9996$).

Tannin Content (TC). The acidic butanol technique was used to quantify the procyanidin content of the extracts.[17] A stock solution of 0.07% (w/v) $FeSO_4 \cdot 7H_2O$ dissolved in 95:5 (v/v) 1-butanol/HCl was prepared. In a test tube, 7 mL of the stock solution and 0.5 mL of the diluted sample were mixed and heated for 50 min at 95 °C. The mixtures were cooled in an ice bath, and the absorbance was measured at 550 nm using a Cintra 20 (GMBH, Germany) double-beam spectrophotometer. A blank reagent was prepared following the same procedure as above, but instead of 95:5 (v/v) butanol/HCl, a mixture of 95:5 (v/v) butanol/H_2O was used. The TC was expressed in cyanidin-3-*O*-glucoside equivalents after the preparation of a standard curve of cyanidin from 0 to 392 mg/L ($r^2 = 0.999$).

Identification and Quantification of Individual Phenolic Compounds. The identification of individual phenolic compounds was performed following the methodology by Guendez et al.[18] After filtration, the extracts were injected with a 45 μm syringe filter directly into a Waters 2695 liquid chromatography apparatus equipped with a Waters 2695 (Waters, Milford, MA) diode array detector set at 280 nm. The separations were made at 40 °C in a LiChrospher RP-18, 5 μm, 250 × 4 mm, column (Merck, Germany) and were protected with a guard column of the same material. Both the column and the guard column were tempered at 40 °C. Eluent A was a solution of 1 mL of 60% perchloric acid in 1 L of type-1 water, and eluent B was MeOH. The flow rate was kept constant at 1 mL/min throughout the analysis. The injections were made with a 20 μL fixed loop. The elution program used was as follows: from 100 to 78% A in 55 min, from 78 to 0% A in 10 min, and then isocratic for another 10 min. Quantification was performed by establishing calibration curves for each compound determined using the standards (GA, $r^2 = 1$; CAT, $r^2 = 1$; EPI, $r^2 = 1$; ProB1, $r^2 = 0.9997$; and ProB2, $r^2 = 0.9999$).

Antioxidant Activity (AA). *ABTS.* The technique to determine the AA was based in the work by Re et al.,[19] with slight modifications. A 7 mM solution of ABTS and a 4.9 mM solution of $K_2S_2O_8$ were made and mixed between 12 and 16 h before use. The mixture was diluted with absolute ethanol to achieve and absorbance of 0.70 ± 0.02 at 734 nm. The diluted ABTS mixture (2970 μL) was put in a 1 cm cuvette, and the absorbance at 734 nm was measured and considered as the initial absorbance. The diluted GSE sample (30 μL) was added, and the absorbance was measured after exactly 6 min. The AA is reported as millimoles of Trolox equivalents per gram of dry extract after the elaboration of a standard curve of Trolox ($r^2 = 0.9985$) based on the reduction percentage on the absorbance at 734 nm after 6 min. All of the absorbance measures were made in a double-beam spectrophotometer Cintra 20 (GMBH, Germany).

DPPH. The antiradical activity of the extract was evaluated on the basis of the technique by Rivero-Pérez et al.[20] A 60 μM methanolic solution of DPPH (2940 μL) was mixed with 60 μL of the extract in a polystyrene cuvette. The absorbance at 515 nm was measured before adding the extract and 60 min after adding it, using a Cintra 20 (GMBH, Germany) double-beam spectrophotometer. The antioxidant activity is reported as millimoles of Trolox equivalents per gram of dry extract after the elaboration of a standard curve of Trolox based on the reduction of the absorbance after 60 min ($r^2 = 0.999$).

Browning Absorbance at 420 nm (A_{420}). The A_{420} of the samples was performed directly on the extracts by measuring the absorbance at 420 nm using a Cintra 20 (GMBH, Germany) double-beam spectrophotometer. The measurement was performed at 420 nm because the aim was to evaluate the browning of the extracts, which is related to the yellowish colors.[21]

Statistical Analysis. Statistical analyses were conducted using SPPS 16.0 (SPPS, Inc., Chicago, IL). Differences among the treatments were determined using an analysis of variance (ANOVA) and a Tukey test, with a confidence level of 95%.

Model Development. Considering that non-enzymatic browning and degradation of some antioxidant compounds do not follow a zero- or first-order behavior,[21,22] the concentration data of the individual phenolic compounds and browning after the thermal treatments (three temperatures at six times) were fitted into two nonlinear nor first-order models using the fitting curve module of Matlab 2010a software (The MathWorks, Inc.).

The Weibull model:[21]

$$C = C_m + (C_0 - C_m)\exp[-(kt)^{\beta}] \quad (1)$$

The combined (combination of zero and first order) model:[22]

$$C = K - (K - C_0)\exp(-k_1 t) \quad (2)$$

where C is the concentration at time t, C_0 is the initial concentration ($t = 0$), k is the formation/degradation or browning rate constant (min^{-1}), C_m is the maximum or minimum concentration, β is the shape constant in the Weibull model (eq 1), and $K = k_0/k_1$ (eq 2). For processes of an increase of the parameter, k_0 can be defined as the

Table 1. Characterization of the GSE[a]

					AA (mmol of Trolox/g of dw)	
parameter	humidity (%)	pH	TPC (mg of GAE/g of dw)[b]	TC (mg of CyE/g of dw)[c]	ABTS	DPPH
amount	6.12 ± 0.09	4.22 ± 0.05	846 ± 8	389 ± 1	7.64 ± 0.17	6.85 ± 0.17

		individual phenolic compounds (mg/g of dw)				
parameter	browning (A_{420})	CAT	EPI	ProB2	ProB1	GA
amount	0.2113 ± 0.0001	68.10 ± 0.37	47.14 ± 0.12	7.54 ± 0.05	4.60 ± 0.07	0.95 ± 0.02

[a] Values represent the means of the results (n = 4) ± the standard deviation. [b] Gallic acid equivalents (GAE) per gram of dry weight (dw). [c] Cyanidin equivalents (CyE) per gram of dry weight (dw).

formation constant, while k_1 is the destruction constant, and vice versa in processes were the parameter decreases.

The obtained rate constants were then fitted to an Arrhenius equation

$$\ln k = \ln A - \frac{E_a}{RT} \quad (3)$$

where T is the absolute temperature (K), E_a is the activation energy (J/mol), R is the universal gas constant (8.314 J mol^{-1} K^{-1}), and A is the pre-exponential factor (min^{-1}).

Model Validation. To validate the prediction models, the natural logarithm of the rate constant (min^{-1}) for the measured parameters on the samples was graphically compared to the ones obtained in independent experiments applying different temperatures (70, 95, and 110 °C) for 10, 25, 40, and 55 min. The comparison was performed using the analysis of fit for new observation included in the fitting curve module of Matlab 2010a software (The MathWorks, Inc.).

RESULTS AND DISCUSSION

Extract Characterization. Table 1 shows the measured parameters to characterize the GSE. The pH of the dissolved GSE is between 4 and 5, which confers more stability to flavan-3-ols and tannins within the extract than more alkaline or acidic values.[4] The TC represents approximately 45% of the TPC, which was approximately 10 times higher than the results obtained by Makris et al.,[2] who used the same methods and units to quantify both parameters. In the case of the AA, it is known that the results using ABTS are higher than the ones using DPPH[23] and the obtained values are similar to those reported by Makris et al.[2]

With regard to the concentration of the individual phenolic compounds measured by HPLC, it is clear that CAT and EPI are the flavan-3-ols with the highest concentration, followed by ProB1 and ProB2 and finally the GA. The order in the concentration of the individual phenolic compounds is similar to the order that Guendez et al.[18] reported, with the difference that, in the work by Guendez et al.,[18] the content of ProB1 is higher than ProB2. The sum of the compounds measured by HPLC represents around 15% of the TPC.

Effect of Thermal Treatments on the GSE Profiles. Panels a–d of Figure 1 show the concentration of the measured flavanols after the thermal treatments. CAT and EPI had a tendency to decrease, while ProB1 and ProB2 had a tendency to increase, with the increment of temperature. In all cases, the changes at 60 °C are barely appreciable. At 90 °C, for CAT and EPI, before reaching the 45 min of heating, the decrease is similar to the one at 60 °C and, for ProB1 and ProB2, the increase is stronger, practically since the beginning of the treatment. For the 120 °C treatment, changes are evident and the concentration of CAT and ProB1 remains stable after 5 min of heating, while the concentration of EPI and ProB2 keeps changing during the whole treatment. It is known that heat induces the acceleration of the oxidation–polymerization process of individual phenolic compounds,[6,24] which explains the rise of the dimers against the decrease of the monomers in the GSE.

The concentration of GA showed an increment with the thermal treatments (Figure 1e). For the 60 °C treatment, the changes on the GA concentration were barely present. In the case of the 90 °C treatment, the tendency of the changes is practically linear. For the 120 °C treatment, the changes took a more sigmoidal shape. The main source of GA units may come from the excision of the gallate group attached to the C ring of the flavonoids [e.g., (−)-epicatechin gallate, (+)-catechin gallate, etc.]. The excised GA unit is called the gallate acid moiety or galloyl moiety. It is known that the release of these units is induce by heat and oxygen[25] and follows the path proposed by Lee et al.[24] Another source of GA may be the hydrolysis of the gallotannins with the increase of the temperature, thus realizing the methyl gallate units.[26] Nevertheless, GSEs have a small amount of gallotannins in their composition,[27] making in this case the hydrolysis of gallotannins a smaller source of GA. The increase in A_{420} is shown in Figure 1f. It can be seen that, in the three studied conditions, the increment in the absorbance is more notorious at the beginning of the heating process, giving the fitting sigmoidal shapes, mainly in the 120 °C treatment. Browning of GSEs after thermal processes was also found by Amendola et al.[28] and Davidov-Pardo et al.[29] The increase in the absorbance at 420 nm could have been due to condensation, polymerization, and oxidation of the polyphenols contained in the sample.[25,28]

ABTS and DPPH measurements after the thermal treatments are shown in panels a and b of Figure 2, respectively. The lack of a clear tendency to increase or decrease on the AA is in agreement with the study by Amendola et al.[28] and made it impossible to fit the results into the kinetics models; the same happened for the TPC and TC (data not shown). The release of these gallate groups can possibly explain the decrease in the AA in the first 5 min, because it is known that the presence of the gallate group joined to the C ring confers a higher AA power to flavan-3-ols.[30] The AA is later stabilized almost to its original value possibly because of the progressive polymerization of catechins, because tannins proved to have more scavenging power that simple phenolic compounds.[31] The fact that these parameters involve many compounds and some of them may be increasing while the others are decreasing results in an apparent thermal stability, which makes it impossible to model.

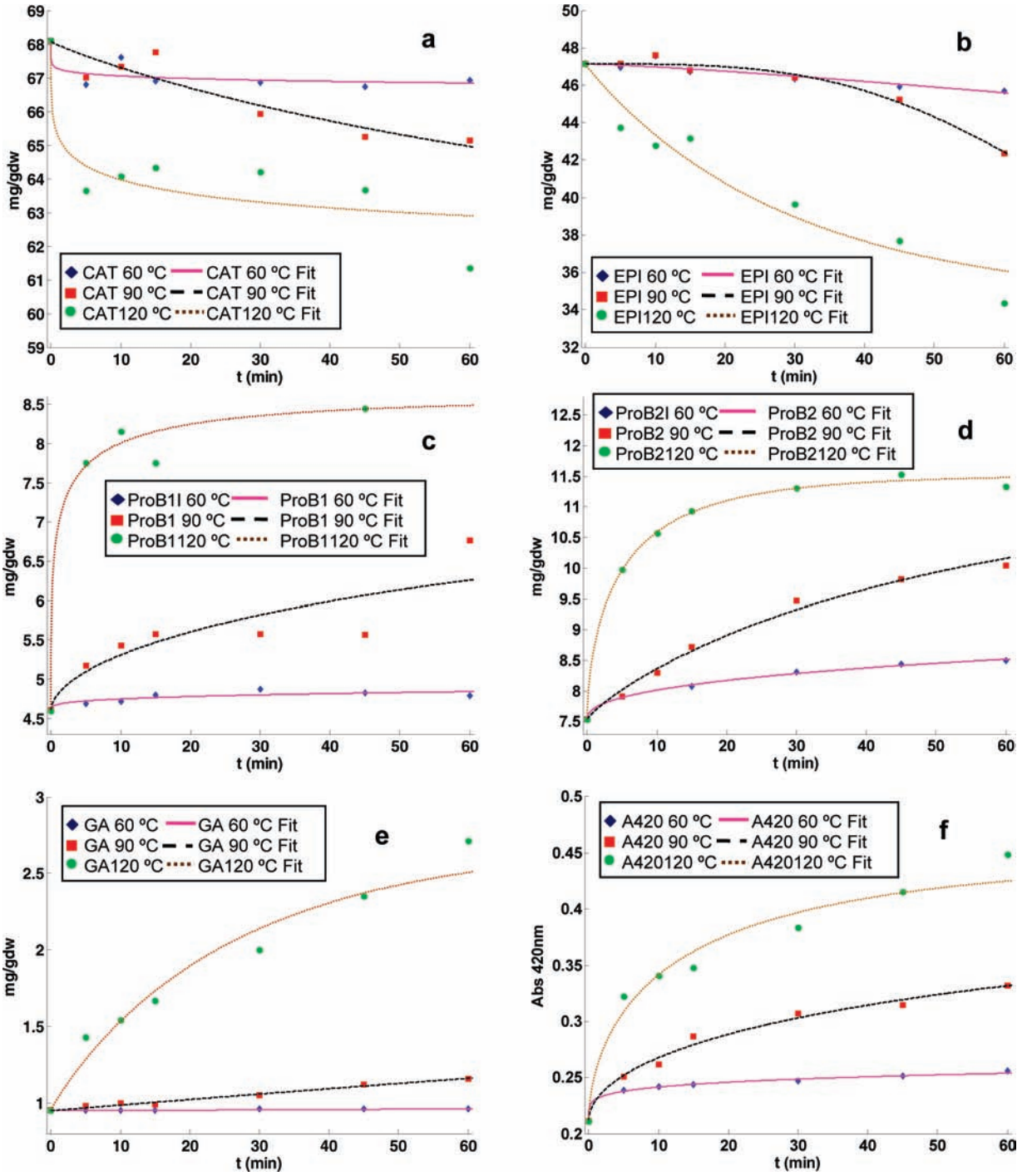

Figure 1. Concentrations and Weibull fitted model after the GSE was submitted to the thermal conditions: (a) CAT, (b) EPI, (c) ProB1, (d) ProB2, (e) GA, and (f) A_{420}.

Comparison between the Mathematical Models. To compare the two models (eqs 1 and 2) used to describe the modification kinetics on the measured parameters of the GSE, the coefficient of determination (r^2) and the mean square error (MSE) values were considered. The Weibull model gave higher r^2 than the combined model, especially when the rate constants were fitted to the Arrhenius equation (Table 2).

When the MSE value is used, MSE values closer to 0 indicate that the fit is more useful for prediction. On the basis of the technique by Vaikousi et al.,[21] a graphical comparison was used to compare the MSE values between the Weibull and combined models (Figure 3). The rate constants calculated by the Weibull model are presented on the x axis, and the differences between the MSE values for the combined model minus the MSE values for the Weibull model are presented on the y axis. The points above the horizontal axis represent cases where the MSE values of the former were higher than those of the latter. Therefore, the Weibull model had lower MSE values in most cases, reinforcing the fact that it had a better fit than the combined model.

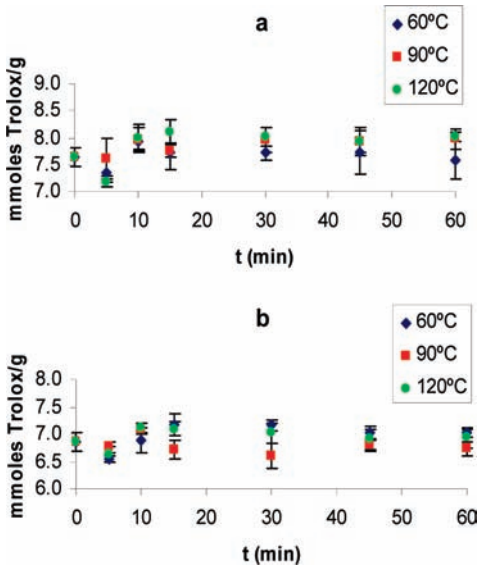

Figure 2. Antioxidant activity of the GSE after all thermal treatments: (a) ABTS and (b) DPPH.

Table 2. Coefficient of Determination (r^2) of the Combined and Weibull Models and the Arrhenius Equation for All of the Parameters and Conditions

parameter	temperature (°C)	combined r^2	Weibull r^2	Arrhenius combined r^2	Arrhenius Weibull r^2
GA	60	0.80	0.80	0.43	0.99
	90	0.98	0.98		
	120	0.96	0.96		
CAT	60	0.68	0.72	0.51	0.88
	90	0.86	0.87		
	120	0.74	0.81		
EPI	60	0.83	0.85	0.66	0.99
	90	0.76	0.98		
	120	0.94	0.93		
ProB1	60	0.90	0.79	0.46	0.98
	90	0.69	0.76		
	120	0.93	0.98		
ProB2	60	0.83	0.99	0.33	0.99
	90	0.99	0.99		
	120	0.99	0.99		
browning	60	0.93	0.99	0.08	0.93
	90	0.98	0.99		
	120	0.92	0.97		

The higher r^2 and lower MSE values of the Weibull model fittings may be due to the use of an incorporated A_{max}, which physical means a maximum increasing/decreasing or browning potential, progressively leading to no further increases or decreases in the concentration or absorbance[21] and to the introduction of the Weibull shape constant, which adjusts the fitting to a sigmoidal shape of the curve. On the other hand, the reason that could explain why the differences in the r^2 values were higher for the Arrhenius equations than for the fitting models may be because probably the changes in the ratio of the rate constants ($K = k_o/k_i$) were not uniform over the studied temperatures,

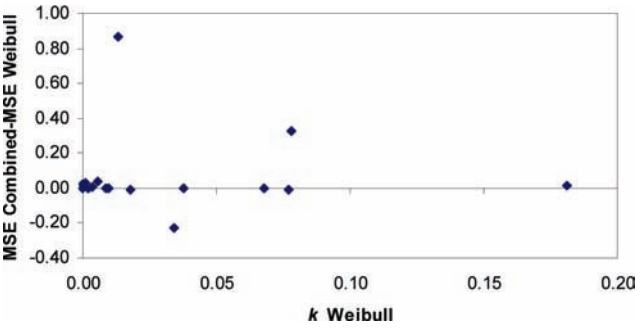

Figure 3. Comparison of the goodness of fit of the Weibull and combined models using the MSE values.

resulting in lower r^2 when fitting k_0 in the Arrhenius equation. In conclusion, the Weibull model was the model selected to evaluate the thermally induced changes on each of the measured parameters.

Activation Energy (E_a) of the Changes in the GSE. After fitting the constant rates of the Weibull model to the Arrhenius equation, the E_a of CAT concentration decrease was 286 kJ/mol, while the E_a for the decrease of EPI was 42 kJ/mol. The latter coincides exactly with the E_a obtained by Wang et al.[14] for the degradation of EGCG and GCG in a green tea extract and is half of the one obtained for the degradation of EGCG by Zimeri and Tong.[32] In flavonols, heat induces changes in the configuration at the C-2 position without changing their optical rotation, with the 2−3 *trans* configuration of CATs being more thermodynamically stable than the 2−3 *cis* configuration of EPIs, making epimerization more frequent than reverse epimerization.[25] The lower thermostability of EPI explains the lower E_a value for its decrease compared to the one for CAT. The E_a for the increase of ProB1 was 249 kJ/mol, and the E_a for the increase of ProB2 was 95 kJ/mol. The fact that ProB2 needs less energy to increase than ProB1 may be related to the lower E_a for the decrease of EPI in comparison to CAT. The higher loss of EPI may not be only because of the epimerization but also because the polymerization of two EPIs requires less energy than the polymerization of one CAT and one EPI, possibly because of its stearic configuration. The E_a of the GA increment was 102 kJ/mol. The E_a for the browning in the GSE was 170 kJ/mol, higher than the ones found by Ibarz et al.[22] and Vaikousi et al.[21] for the browning of pear purée, orange juice, and apple juice, respectively.

Model Validation. To validate the results obtained after modeling the changes in the GSE, the natural logarithms of the rate constants (min^{-1}) for the measured parameters were graphically compared to the ones obtained in independent experiments applying different temperatures (70, 95, and 110 °C) for 10, 25, 40, and 55 min (panels a−f of Figure 4). This comparison was made by means of the analysis of fit for the new observation included in the fitting curve module of Matlab 2010a software (The MathWorks, Inc.). The Arrhenius equations of the original and the validation experiments were fitted to the new observation values of $1/T$. In all cases, both fitted Arrhenius equations and the natural logarithms of the rate constants (min^{-1}) for the validation experiments fell between the 95% confidence boundaries.

It can be said that among the reactions and changes that occur when a GSE is heated, three were evident: (1) release of GA units, (2) epimerization and polymerization of catechins, and (3) darkening of the sample.

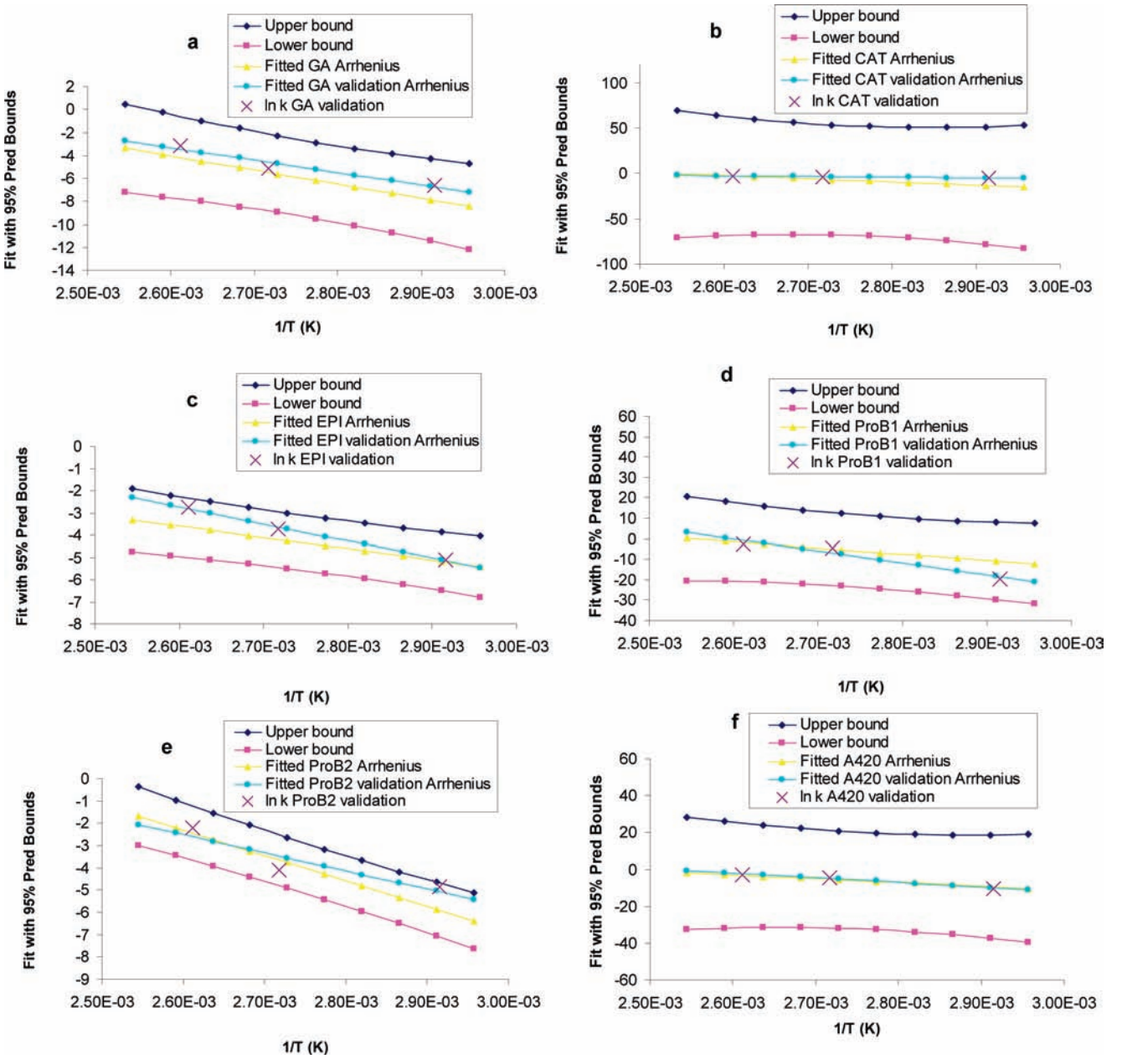

Figure 4. Arrhenius analysis of fit for the new observation: (a) GA, (b) CAT, (c) EPI, (d) ProB1, (e) ProB2, and (f) browning.

It can also be concluded that, when a Weibull equation is used, it is possible to model and obtain fittings of the changes in the concentration of GA, CAT, EPI, ProB1, and ProB2 and the increment of A_{420} of a commercial GSE after heating it, with $r^2 > 0.7$. It is also possible to fit the rate constants of those changes in the Arrhenius equation and obtain the E_a, with coefficients of determination of $r^2 > 0.88$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Table with coefficients k (min^{-1}) and β of the Weibull equation for each parameter and table with ln A (min^{-1}) and E_a (J/mol) of the Arrhenius equation for each parameter. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

GSE, grape seed extract; GA, gallic acid; ProB1, procyanidin B1; ProB2, procyanidin B2; CAT, (+)-catechin; EPI, (−)-epicatechin; EGCG, (−)-epigallocatechin gallate; GCG, gallocatechin gallate; TPC, total

phenolic content; TC, tannin content; AA, antioxidant activity; A_{420}, absorbance at 420 nm

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