Sugar Conversion Induced by the Application of Heat to Grape Must

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Two lots of the grape Trebbiano cultivar were harvested from the same vineyard 15 days apart, and their musts were cooked in an open stainless steel pan directly heated by fire; the kinetics of formation or disappearance of key constituents was then monitored for at least 16 h. From an engineering standpoint, the vessel behaved like a nonisothermal batch reactor in which the volume of the grape must necessarily decreased while its composition changed profoundly as a result of chemical reactions. Brix, total titratable acids, acetic acid, malic acid, lactic acid (D and L), pH, water activity, 5-HMF, and phenolic and radical-scavenging compounds were proposed as markers of the extent of cooking for which water vaporization and sugar degradation were identified as the two main driving factors. Acid-catalyzed dehydration was hypothesized as the predominant mechanism for sugar degradation, assuming a direct role of water vaporization; however, contributions of Maillard degradation pathways and other parallel reversible reactions were also hypothesized. Fractional conversion of 5-HMF and radical-scavenging compounds were proposed as quantitative markers for the extent of sugar degradation at, respectively, the early and advanced stages of cooking. Selectivity indices were also proposed as a performance criterion to design cooking processes in relation to sugar degradation.

INTRODUCTION

Grape must and concentrated grape must are used as starting materials for the production of several Italian foodstuffs, often after deep concentration caused by heating. Thermally concentrated grape must obtained at atmospheric pressure using heated open pans is used in the production of the traditional balsamic vinegars of Modena and Reggio Emilia provinces, which have recently earned protection of their appellation by the European Community (G.U. n.124 del 30/05/2000). There is evidence that during heating grape juice undergoes extensive changes of physical properties such as density, viscosity, specific heat, and coefficient of thermal expansion (*1*, *2*) and that these changes affect the rheology of boiling juice (*3*, *4*). Moreover, the high temperatures and long heating times induce thermal degradation of the sugars, which within aqueous solution are unstable and undergo a number of reactions such as mutarotation, enolization and isomerization, anhydride formation, and polymerization. Because such reactions are reversible, the starting sugar can be regenerated via enediol formation (*5*). Glucose has competing reaction pathways that lead to the formation of byproduct: in one pathway; dehydration forms non-furan cyclic ethers; in another, C−C bond scission occurs through reverse aldol condensation (*6*). The type and magnitude of degradation products will depend on the starting reagent condition (*7*). Acetic, formic, and D/L-lactic acids were identified at the end of thermal degradation of sugar

solutions (*8*), and a reaction scheme was proposed by Ginz et al. (*9*). Sugar degradation may result in browning of solutions with polymeric compounds as the ultimate product of degradation, generally known as "melanoidins", involving the formation of 5-(hydroxymethyl)-2-furancarboxaldehyde (5-HMF) as intermediate. In acidic solutions, glucose and fructose convert via acid-catalyzed mechanisms, the first step of which consists of a structural rearrangement of the sugar backbone into an intermediate 1,2-enediolic form, which rapidly eliminates water molecules before producing 5-HMF (*10*). From a safety perspective, sugar degradation is responsible for some important health concerns related to the accumulation of 1 HMF and other furanic congeners in foods (*11*). 5-HMF is widely recognized as a potential cytotoxic, genotoxic, and tumorigenic agent: law fixes a limit of 25 ppm for the rectified grape must (Regulation (EC) No. 1493/99). 5-HMF can be formed from dehydration of fructose in its furanose form through a series of cyclic furan intermediates (*12*) or through an acyclic mechanism proceeding through an enediol pathway (*6*). However, 5-HMF is also known as a major intermediate of the Maillard reaction: in the presence of aminic group donors, sugars first rearrange into Amadori compounds and then produce 5-HMF via cyclization and condensation reactions (*13*). Some different grape varieties and cooking technologies were investigated for the formation of furanic congeners during must cooking (*14*). The conversion of fructose and glucose under accelerated storage tests for the accumulation of 5-HMF and brown pigment formation (BPF) was studied in systems simulating grape musts containing more than one sugar and

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amino acids (*7*). In these experiments, authors found that the accumulations of both the 5-HMF and BPF in the presence of glutamine or arginine were higher than in the model systems containing only sugars at pH 3.5. The effect of the amino acids was additive, and the rate of conversion followed the Arrhenius law in the range of 55−75 °C. The first systematic study on the kinetics of HMF formation under cooking was carried out in a model system that simulated the acidic conditions of the grape must (*15*). The authors evaluated the single contributions of glucose and fructose concentration as well as of water activity and pH level, treating all of these properties as independent variables. The authors supposed that both the glucose and fructose converted through acid-catalyzed dehydration and cyclization mechanisms and the rate of 5-HMF formation are functions of water activity and, to a lesser extent, of sugar concentration and pH value. The purpose of this work was to analyze heat-induced changes in the main physical properties of the grape must and in sugar conversion, taking into account the initial composition of grape juice.

MATERIALS AND METHODS

Must Preparation and Cooking Experiments and Extent of Cooking Evaluation. Two lots of cv. Trebbiano grapes, growing in the same Reggio Emilia vineyard, were harvested with 2 weeks of each other (on October 1 and 15, 2009 vintage). Both lots were washed and crushed by means of the traditional equipment used for wine production and then cooked in two different batches. In the following, the must obtained from short- and long-ripened grapes will be indicated as "must from SRG" and "must from LRG", respectively.

Cooking was carried out in an open stainless steel vessel (diameter = 110 cm, height = 130 cm) fitted with a mechanical stirrer and operating at atmospheric pressure. A butane burner was used as a source of fire directly in contact with the bottom of the vessel, and a coaxial stainless steel jacket was used to concentrate the hot air surrounding the vessel themselves. The weight of the fresh grape must was fixed at 100 kg for both batches, and cooking was monitored for a minimum of 16 h.

From an engineering point of view, the progress of the cooking process can be quantitatively determined by measuring all extensive properties able to describe the cumulative effects as dictated by mass and energy balances into the grape must under process. The amounts of D-glucose and D-fructose (the main sugars of the grape), reducing sugars, total dry matter, D-malic acid, lactic acids (D and L isomers), acetic acid, overall titratable acids, overall phenolic compounds, overall radical-scavenging compounds, and 5-HMF are additive in character and depend on both the size and amount of the matter of interest and were therefore used as extensive properties. Some bulk properties of the grape musts were also measured to evaluate the cumulative effects of cooking at a macroscopic scale: in contrast with the extensive properties, they are nonadditive (intensive) in character and do not depend on size or amount of matter under process. In particular, the pH value, water activity, refractive index, and density were measured throughout cooking because they are widely believed to play a role in sugar degradation reactions in aqueous media. All grape samples were withdrawn at irregular intervals throughout cooking, sealed in open glass vials, rapidly cooled in an ice−water bath, diluted with distilled water (1:10 p/v) to prevent solute precipitation, and, finally, stored under freezing conditions until analysis.

Chemical Measurements. *Sugars and Organic Acids.* D-Glucose (D-Glu) and D-fructose (D-Fru) as well as acetic acid (AcH), D-malic acid (D-MaH), D-lactic acid (D-LaH), and L-lactic acid (L-LaH) were determined using the enzymatic method recommended by the Compendium of International Methods of Wine Analysis -OIV 2006 -MA-E-AS311-02-GLUFRU, with few modifications. Enzymatic kits supplied by Roche (Darmstadt, Germany) were used, together with a spectrophotometer model V-550 (Jasco, Tokyo, Japan) equipped with a UV−vis detector for the absorbance readings at 340 nm that were converted in concentration data (weight to volume) according to the response provided by standard solutions. All HPLC-grade standards were furnished by Aldrich Chemical Co. (Milwaukee, WI). Due to the high concentration of both sugars, samples of fresh musts and cooked musts had been previously diluted with distilled water at a weight ratio of 1:1000, discolored on active charcoal, and finally filtered with cellulose nitrate with 0.2 μm cutoff.

Reducing Sugars (*RS*). The assay procedure used in this work to determine the amount of RS was based on Fehling's method as specified by ref *16*.

5-HMF. The high-performance liquid chromatography (HPLC) method appointed by Yuan and Chen (1999) (*17*) was used with some modifications to determine the concentration of 5-HMF. For this purpose, a Jasco HPLC system equipped with a UV−vis detector (UV−vis-2031 Plus, Jasco Corp.), a cation-exchange column AminexXM-87H (Bio-Rad, Hercules, CA; 300 mm × 7.8 mm), a volumetric injector Rheodyne (Cotati, CA), and an oven with temperature control were used. The operative conditions were as follows: 30 °C for the column, 20 μL the injected volume of sample, and 0.5 mL/min the volumetric flux of the mobile phase (aqueous solution of sulfuric acid, 1 mM). Absorbance readings at 280 nm were acquired, and the concentration of 5-HMF was obtained from calibration against external standard solutions. HPLC-grade 5-HMF was furnished by Aldrich Chemical Co.

Titratable Acids and pH. The overall amount of titratable acids was expressed as milliequivalents of 0.1 N NaOH aqueous solution used to neutralize 100 g of grape musts at pH 7. A Crison pH-meter (Instruments Lab Control, Reggio Emilia, Italy) was used for measurements of pH values.

Total Dry Matter (*DM*). The amount of total DM was determined according to the official method recommended by the AOAC (1995). DM determination was performed using an AMB 50 moisture balance (Adam Equipment Co. Ltd., Milton Keynes, U.K.), and results are expressed as % w/w.

Total Phenolic Compounds (*TPC*). The TPC was determined using the method proposed by Stevanato et al. (*18*), with some minor variations; results were expressed in milligrams of catechin equivalents per liter according to an external calibration curve. This method is sensitive against all molecules having phenolic rings and able to give reactive aryloxy radicals and, contrary to the widely used Folin−Ciocalteu method, is not affected by Maillard reaction products, ascorbate, citrate, or sulfite (*19*). With this aim, 0.1 mL of sample or standard solutions diluted 1:50 v/v was mixed into 3 mL of 0.1 M potassium phosphate solution buffered at pH 8 containing 3 mM 4-aminoantipirine, 2 mM H_2O_2, and 10 U of horseradish peroxidase (HRP). Absorbance readings were done at 500 nm 15 min after mixing (i.e., at the end point of enzymatic reaction) using a double-beam Jasco spectrophotometer equipped with 0.1 cm optical path quartz cuvettes. Catechin standard solutions were prepared by dissolving pure catechin in HPLC-grade water, obtaining concentrations ranging from 0.1 to 3 mg/100 mL.

Total Radical-Scavenging Compounds. The method proposed by Re et al. (*20*) was used to determine the overall amount of compounds with radical-scavenging activity (RSA). ABTS was dissolved in distilled water to 14 mM concentration. ABTS radical cation (ABTS$^{•+}$) was produced by reacting to the ratio of 1:1 ABTS stock solution with 4.9 mM potassium persulfate solution and leaving the mixture to stand in the dark at room temperature for 12−16 h before use. In the assay, 40 μL of appropriately diluted sample was added to 1960 μL of the resulting blue-green ABTS, and concentration data were expressed in milligrams of vitamin C equivalents per liter of grape must.

Physical Measurements. *Refractive Index.* The refractive index was determined using an Abbe's refractometer model 2WA (Alessandrini, Italy) according to the method recommended by the Compendium of International Methods of Wine Analysis (OIV 2006 - MA-E-AS2-02-SUCREF) with a few modifications. Although the refractometric method is considered not selective against the substances dissolved in complex aqueous solutions, in this work the Brix degree calculated from refractive index measurements was used to indicate the concentration, expressed in percentage by weight, of reducing sugars as well as of the sum of glucose and fructose, assuming as negligible the refractive contribution arising from all other compounds and from the absorbing contribution arising from all colored substances present in the grape musts. Such assumptions were supported by a recent study focused on the use of refractometric measurements in grape musts and some derivatives (*21*).

Density. Density was determined at 20 °C according to the method recommended by the Compendium of International Methods of Wine Analysis (OIV 2006 - MA-E-AS2-01-MASVOL).

Water Activity. Water activity (a_w) was determined under noninvasive conditions using Novasina LabMaster-aw (Lachen SZ, Switzerland),

Table 1. E-PCA Statistics Associated with the First Two Principal Factors Relating to All of the Must Properties Measured throughout Cooking

variable contribution			factor coordinates			communalities		
cooking marker	factor 1	factor 2	cooking marker	factor 1	factor 2	cooking marker	factor 1	factor 2
				Must from SRG				
Brix	0.0738	0.0024	a_w	0.9703	0.0746	Brix	0.9816	0.9843
RS	0.0736	0.0030	pH	0.9346	0.1586	RS	0.9794	0.9828
TitAc	0.0735	0.0018	AcH	0.3417	−0.9064	TitAc	0.9788	0.9808
density	0.0734	0.0014	L-LaH	−0.6853	0.0937	density	0.9767	0.9783
DE	0.0724	0.0000	D-LaH	−0.8207	−0.0017	DE	0.9630	0.9630
D-Fru	0.0721	0.0017	polyph	−0.8281	0.4502	D-Fru	0.9593	0.9612
D-MaH	0.0717	0.0076	RSA	−0.9414	0.0928	D-MaH	0.9541	0.9627
D-Glu	0.0711	0.0173	5-HMF	−0.9608	−0.1510	D-Glu	0.9464	0.9660
a_w	0.0707	0.0049	D-Glu	−0.9728	−0.1403	a_w	0.9415	0.9470
5-HMF	0.0694	0.0201	D-MaH	−0.9768	−0.0926	5-HMF	0.9231	0.9459
RSA	0.0666	0.0776	D-Fru	−0.9794	−0.0435	RSA	0.8862	0.8948
pH	0.0656	0.0221	DE	−0.9813	−0.0041	pH	0.8734	0.8985
polyph	0.0515	0.1786	density	−0.9883	−0.0400	polyph	0.6857	0.8884
D-LaH	0.0506	0.0001	TitAc	−0.9893	−0.0446	D-LaH	0.6735	0.6735
L-LaH	0.0353	0.0077	RS	−0.9896	−0.0587	L-LaH	0.4697	0.4784
AcH	0.0088	0.7238	Brix	−0.9907	−0.0524	AcH	0.1168	0.9384
				Must from LRG				
TitAc	0.0700	0.0007	a_w	0.9790	−0.0140	TitAc	0.9933	0.9940
RS	0.0699	0.0015	pH	0.8295	0.2707	RS	0.9931	0.9947
Brix	0.0699	0.0030	AcH	−0.3302	0.9316	Brix	0.9927	0.9959
density	0.0692	0.0019	polyph	−0.9282	−0.2454	density	0.9830	0.9851
5-HMF	0.0684	0.0001	RSA	−0.9683	−0.0887	5-HMF	0.9716	0.9717
a_w	0.0675	0.0002	ES	−0.9707	−0.0698	a_w	0.9585	0.9587
D-Glu	0.0675	0.0055	L-LaH	−0.9712	0.1114	D-Glu	0.9581	0.9640
D-Frau	0.0674	0.0062	D-MaH	−0.9764	0.0792	D-Fru	0.9576	0.9641
D-LaH	0.0673	0.0106	D-LaH	−0.9775	0.1061	D-LaH	0.9554	0.9667
D-MaH	0.0672	0.0059	D-Fru	−0.9786	0.0809	D-MaH	0.9534	0.9597
L-LaH	0.0664	0.0117	D-Glu	−0.9788	0.0766	L-LaH	0.9433	0.9557
DE	0.0664	0.0046	5-HMF	−0.9857	0.0092	DE	0.9422	0.9470
RSA	0.0660	0.0774	density	−0.9915	−0.0451	RSA	0.9376	0.9455
polyph	0.0607	0.0566	Brix	−0.9963	−0.0565	polyph	0.8615	0.9217
pH	0.0485	0.0689	RS	−0.9965	−0.0396	pH	0.6881	0.7614
AcH	0.0077	0.8154	TitAc	−0.9966	−0.0275	AcH	0.1090	0.9768

a resistive electrolytic humidity measuring system, able to give an accuracy of ±0.002 a_w unit and equipped with a Novasina eVC-21 filter (Lachen SZ), a chemical filter able to entrap volatile compounds including acetic acid. a_w values were registered when there was confidence that equilibrium had been reached, a process that never took < 1 h.

Data Processing and Statistical Analysis. Using Statistica 7.0 (StatSoft Inc., Tulsa, OK), both the extensive and intensive properties of the grape musts were used as explorative/descriptive variables aiming to give a simplified picture of the whole process of cooking through explorative principal component analysis (E-PCA) and descriptive principal component analysis (D-PCA). To eliminate the scaling effect, the relative changes were calculated by dividing the actual value $I(t)$, measured at the time of heating (t), by the respective initial value $I(0)$, measured in the fresh must (zero time of heating); these were then added, as fresh input, into the two statistical procedures. Using proFit 6.1.1 (Quantum Soft, Uetikon am See, Switzerland), time-dependent data were fitted by nth-order polynomial functions aiming to evaluate the rate of chemical conversion taking place during cooking. The quality of fit was also evaluated. Finally, the rate of sugar conversion was evaluated against the bulk properties as fixed by cooking.

RESULTS AND DISCUSSION

Engineering Factors Driving Cooking. E-PCA was performed first, to highlight the hidden relationships among all investigated properties of the grape musts as fixed by cooking. With this aim, the correlation matrix was utilized to reduce the dimensions of the original space of variables starting with an eigenvalue of > 1. Only the first two principal factors were retained for the two batches of

cooking; that is, factor 1 accounted for 83.18 or 88.74%, and factor 2 for 7.09 or 6.65%, of the data variance observed. Some key E-PCA statistics associated with factors 1 and 2 including "variable contribution", "factor coordinates", and "communalities" were calculated (**Table 1**) and used to get physical meaning for the two retained principal factors. According to the greatest values for all of these statistics, two main engineering driving factors were recognized for the cooking process: the first was water vaporization, resulting in must volume lowering as well as in solute concentration; the second was the chemical degradation of sugars. The relative increase of 5-HMF concentration cannot be explained by volume lowering only. Consequently, factor 1 was labeled "solute concentration" and factor 2 "sugar degradation". The relative changes in Brix, total titratable acids, acetic acid, malic acid, lactic acid (D and L), pH, water activity, 5-HMF, and phenolic and radical-scavenging compounds were then selected as markers for the cooking extent (MCE). The cumulative effects of cooking can be followed graphically in **Figure 1**. Solute concentration and sugar degradation explain not only the 77.34 and 85.22% figures but also the 10.01 and 9.50% experimental variance for the two cooking batches.

Solute Concentration and Browning. The vessel used for cooking behaved as an un-steady-state batch reactor in which water vaporization takes place as the main physical factor and sugar degradation as the main chemical factor, driving cooking simultaneously. Working at atmospheric pressure, water vaporization from the must surface is driven by the relative humidity of the air
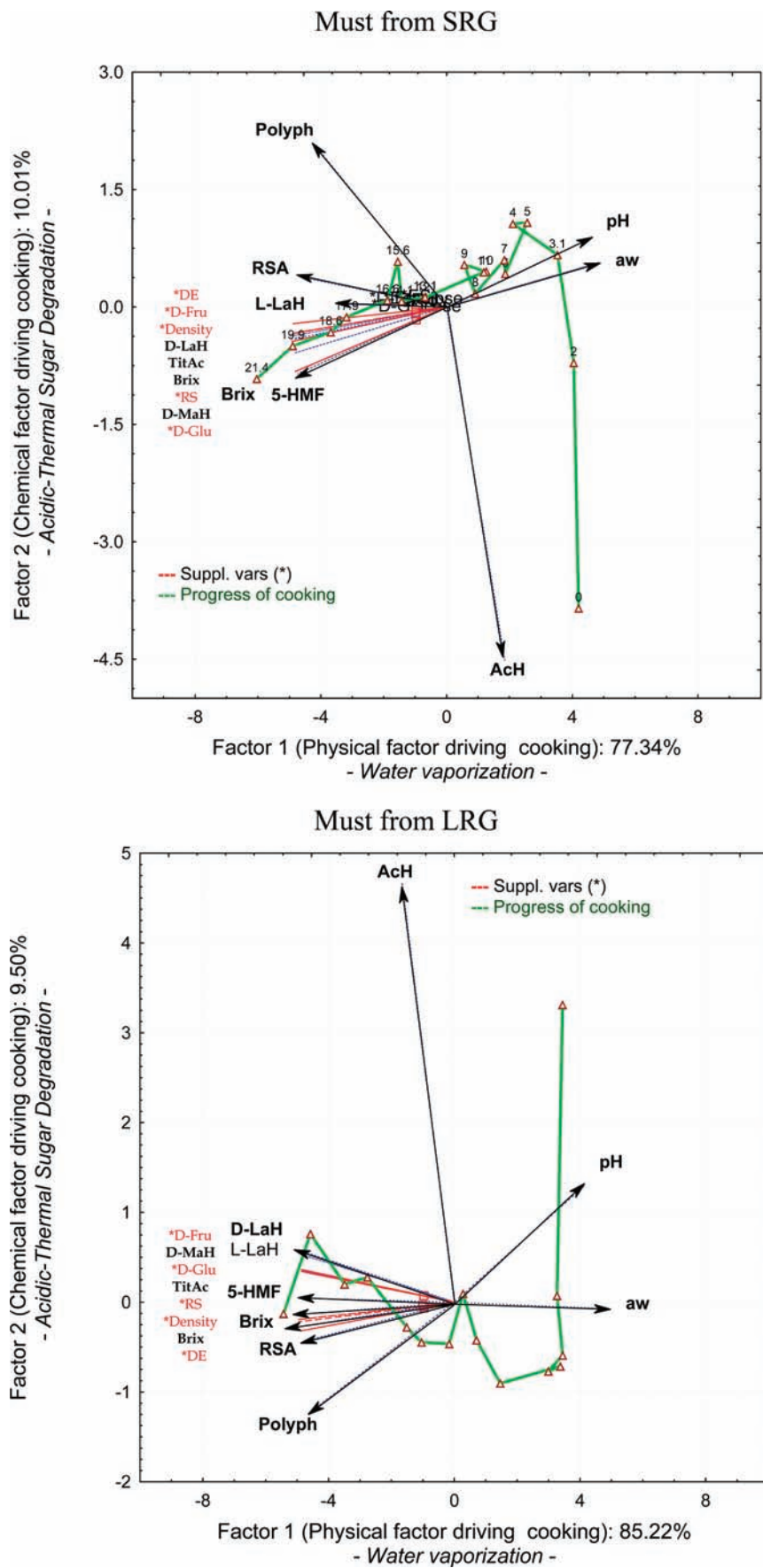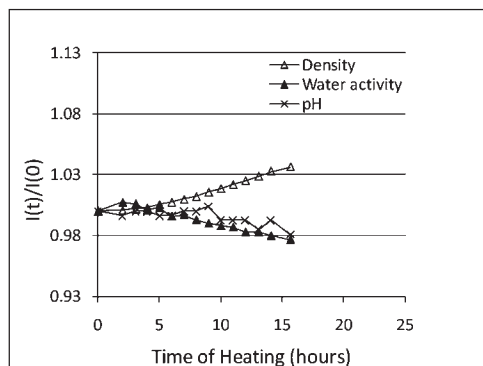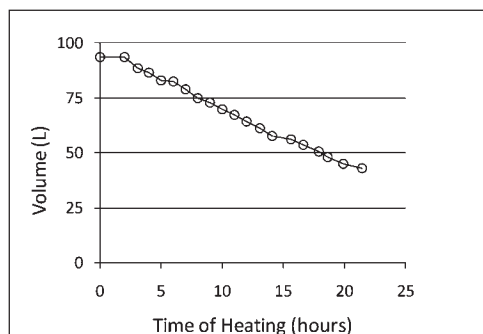
Figure 1. D-PCA results highlight the two engineering factors driving cooking: a first factor of physical nature, that is, the water vaporization; and a second first factor of chemical nature, that is, sugar degradation reactions. The time of heating (hours) is reported as a numerical label along with the score plots.

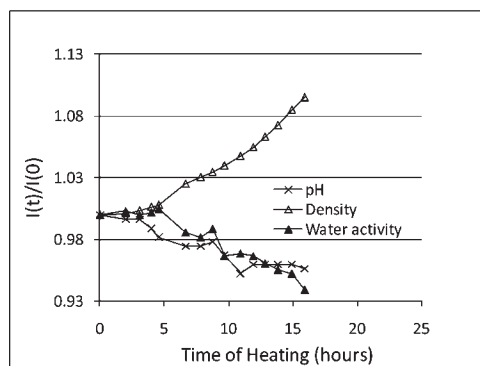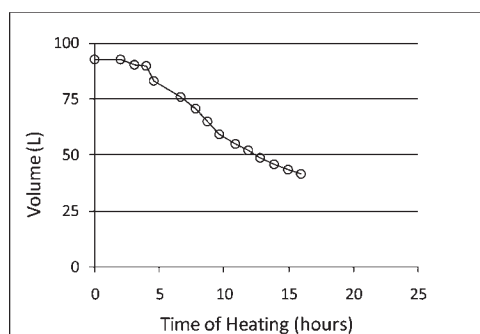Must from SRG   Must from LRG

Figure 2. Extent of the overall cooking process as described by time course of the proposed markers. Markers account simultaneously for both the water vaporization and sugar degradation reactions.

surrounding the cooking system, which in this work was not controlled. It is widely accepted that water vaporization involves simultaneous heat and mass transfer and depends on the amount of heat supplied to vaporize the water and remove vapor from the vicinity of the surface at which vaporization takes place. However, in this case, sugar degradation reactions caused the main changes in the grape must composition, with different kinetics between the two types of grape must. As a consequence, chemical conversion would be expected to play a role in water vaporization kinetics. Such behavior could be attributed to an increase of the bulk ability to bind the free water, that is, the hygroscopic capacity of the grape must, as caused by sugar concentration through cooking.

Although water vaporization was the most evident effect of cooking, heat supplied under our cooking experiments caused the formation of fouling materials, mainly foams and natural colloids. However, because of the frequent cleaning interventions aimed at mechanical elimination of fouling materials, and because of efficient stirring, mass and heat transfer were assumed to be uniform at any time inside the batch volume upon cooking. This hypothesis was supported by the fact that the time course of temperature data was the same for both grape must types (data not shown). As a consequence, the volume was considered to be homogeneous, and the same boundary conditions for heat supply were assumed for the two batches of cooking. Both the extent and rate of changes observed in the bulk properties of water activity and pH as well as in density were mainly attributed to the solute concentration caused by the water vaporization process throughout cooking. Deep changes in color under cooking conditions are also important evidence. Differences observed between the two batches were attributed to the different starting compositions, which caused different vaporization rates throughout cooking. The data showed in fact that the initial volumes of SRG and LRG musts were lowered to about 42 and 55%, respectively, despite either the starting and final bulk temperatures were 25 and 95 °C

for both batches (**Figure 2**). Changes in the composition and physical properties were recorded by means of a visual image (**Figure 3**). Data from the two cooking batches were standardized and centered to eliminate any scaling effect and also the heterogeneity of the original data variance (the zero value represents the average levels for each measured property). As can be inferred from the data, cooking rapidly stopped all enzymatic browning caused by the polyphenol oxidase known to occur in the fresh grape juice; next, it promoted bulk discoloration and clarification, probably due to the thermal deactivation of browning enzymes; and, finally, it produced progressive nonenzymatic browning. The concentration of acetic acid underwent a rapid decrease in the early stages of cooking while, at the same time, D-glucose, D-fructose, reducing sugars, titratable acids, malic acid, and lactic acid (both D and L isomers) concentrated together, throughout cooking, with phenolic and radical-scavenging compounds. Conversely, 5-HMF concentrated, in line with a power law, after a lag period; later, nonenzymatic browning did take place, although with different starting times between the two grape musts under investigation. It was hypothesized that during the lag period, sugar degradation started with the accumulation of the uncolored precursors of 5-HMF and then progressed with the formation of 5-HMF and browning compounds. A similar trend for HMF accumulation against heating time was found in systems simulating grape juice (*7*).

Extent and Selectivity of Chemical Conversions. The final amount of furans including 5-HMF and formation kinetics are topics of great interest for cooked must production, from a safety perspective. Under the experimental conditions, the concentrations of 5-HMF after 16 h of cooking was 433.5 and 929 ppm in the short- and long-ripened grape musts, respectively. When the cooking time was prolonged to 20 h, must from SRG accumulated 1029 ppm of 5-HMF. Because water vaporization contributes greatly to the solute concentration, its contribution needed to be individuated before analysis could be made of all chemical
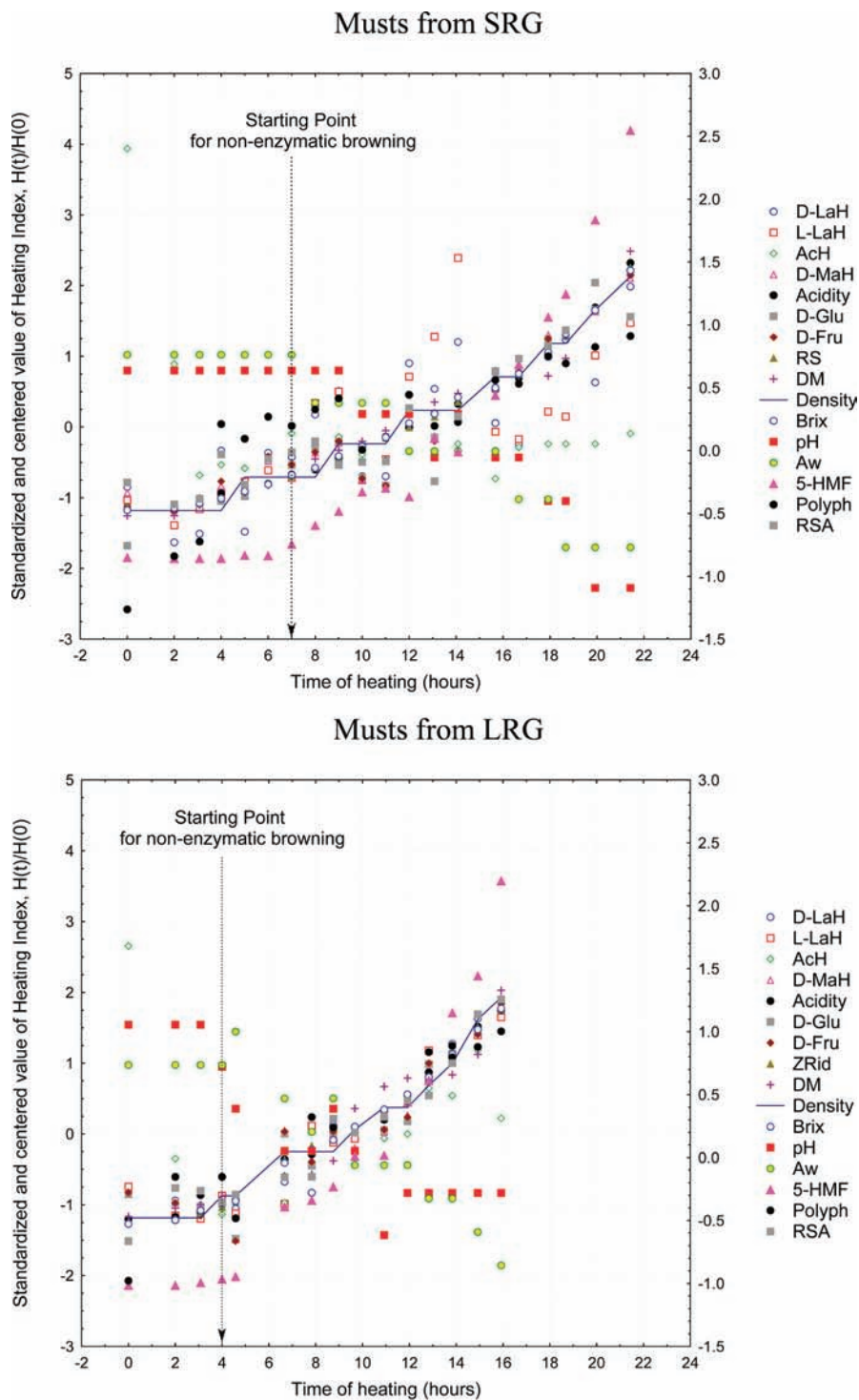
Figure 3. Role of cooking on both the enzymatic and nonenzymatic color changes. Experimental data were standardized and centered, thereby aiming to eliminate scaling effect and heterogeneity of the data variance, allowing comparative purposes among the must properties. The photograph presents a visual image of the color changes in the SRG grape must throughout cooking.

conversion induced by cooking. With this aim, the actual fractional mass for each investigated extensive property $I(t)$ was calculated by coupling the two following equations:

$$\frac{V(t)}{V(0)} = \frac{1}{d(t)} \times \frac{DM(t)}{DM(0)} \qquad (1)$$

and

$$\zeta_t^I = \frac{I(t) \times \left[\dfrac{V(t)}{V(0)}\right] - I(0)}{I(0)} \qquad (2)$$

In these equations, (0) and (t) refer to an extensive property measured in the fresh musts (zero cooking time) and at a given time (t) of cooking. V is the volume of the grape must; DM is the concentration of total dry matter; $V(t)/V(0)$ is the lowering of the product volume as dictated by water vaporization. Equation 1 was obtained by performing the overall mass balance analysis through the must volume under cooking with the following initial and boundary conditions: the initial mass of the two grape musts entering the vessel was fixed at 100 kg; vaporized water was the only stream leaving the must volume (the amount of fouling materials was negligible, < 1% in weight with respect to the grape must bulk); and both the volume and density were time-dependent variables. Total dry matter was taken as a constant basis for mass calculations for all extensive properties. $\zeta_t^{[I]}$, in the following "fractional conversion level" at a given time of cooking (t), is equal to 0 at zero time of heating (of the fresh must). It is worth noting that $\zeta_t^{[I]}$ treated all of the individual compounds [I] indifferently as reactants, intermediates, or products eventually involved in a chemical reaction: a negative value indicates the reactant role (consumption of the compound), whereas a positive value indicates the product role (new formation of the compound).

The time course of fractional conversion levels for sugars and organic acids was as reported in **Figure 4**. As can be inferred, cooking allowed chemical conversion of D-glucose, D-fructose, total titratable acids, and individual acids with different kinetics between the musts from short- and long-ripened grapes. All underwent a negative conversion (consumption) at the early stage of cooking and a complex time-dependent conversion at the advanced stages. Fractional conversion of 5-HMF is plotted against the time of cooking in **Figure 5**. Data showed that chemical conversion of 5-HMF started to form and accumulate after the lag period; however, fractional conversion increased with cooking time, according to a linear trend different from that observed for 5-HMF concentration against the cooking time (data not shown). The linear trend indicates zero-order kinetics of 5-HMF formation. Kinetics of HMF formation has also been investigated by other studies (*22*), and it is reported that this reaction fitted zero-order, half-order, first-order, and second-order kinetic models. **Table 2** reports the concentration both at zero time and after 16 h of cooking for all of the investigated extensive properties, plus fractional conversion levels. Whereas reducing sugars and Brix always showed a negative conversion after 16 h of cooking, the opposite behavior was observed for the D-fructose in the must from SRG, where its positive conversion indicates formation. D-Lactic acid and L-lactic acid showed a negative conversion in the must from SRG and a positive one in the must from LRG. This means that both the sugars and the organic acids may act not only as reactant but also as product under our conditions. At the same time, the fractional conversion level of acetic acid rapidly decreased in the early stages of cooking, probably as a result of direct evaporation due to its low boiling temperature; however, the possibility of its direct formation
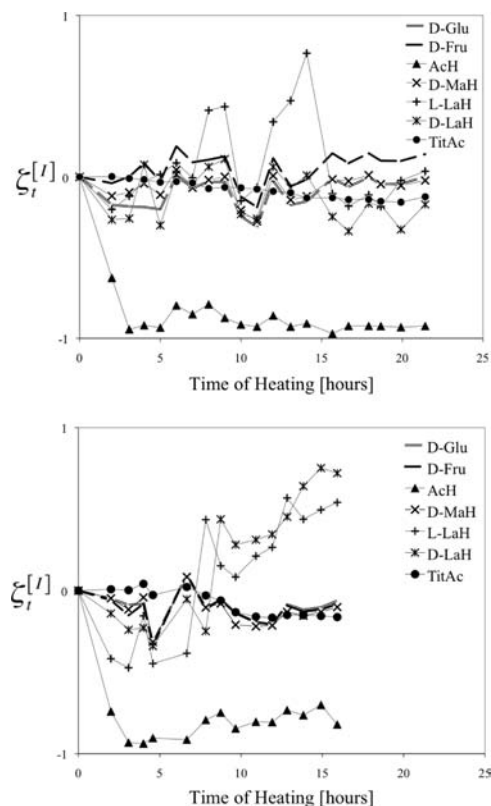

Figure 4. Extent of chemical conversions related to sugar degradation process. Fractional conversion of individual compounds was treated as a quantitative index of the extent of chemical reactions and was calculated by taking into account volume lowering upon cooking.

through cooking cannot be excluded. Both 5-HMF and radical scavenging compounds converted positively in both cooking batches, with a greater extent of accumulation in the must from LRG after 16 h of cooking but with a lower level of conversion with respect to the starting level. The opposite behavior was observed for phenolic compounds: they converted positively in the must from SRG and negatively in the must from LRG.

We hypothesize the presence of some reversible reactions such as isomerization, enolization, retro-aldolization, and polymerization, parallel to sugar degradation, in explaining the opposite conversion of glucose, fructose, malic acid, and phenolic compounds. Some experimental evidence reported in the literature supports our hypothesis. As far as the sugars are concerned, it is known that concentrated solutions of glucose convert partially in a complex mixture of reversion products including fructose, mannose, and oligosaccharides (*5*). A reversion mixture including isomaltose, gentiobiose, cellobiose, and maltose has been isolated in thermal-acid treatment of D-glucose (*23*). In the presence of hydrochloric acids and at 98 °C, glucose forms extensively di-, tri-, and tetra-saccharides, whereas D-fructose showed no formation of such reversion products (*24*). Size exclusion chromatography analysis of cooked musts from cv. Trebbiano, the grape used in this work, confirmed the presence of refractive compounds with a molecular size broadly dispersed around that of the glucose and fructose (*25*). As far as the organic acids are concerned, thermal degradation of preformed Amadori compounds may result in a drop of pH and may produce some organic acids including acetic and lactic acids as well as parent sugars such as glucose and mannose through enolization or retro-aldolization mechanisms (*26*). Under acidic conditions, amino acids previously included in the Amadori compound are released, whereas the rate and types of degradation

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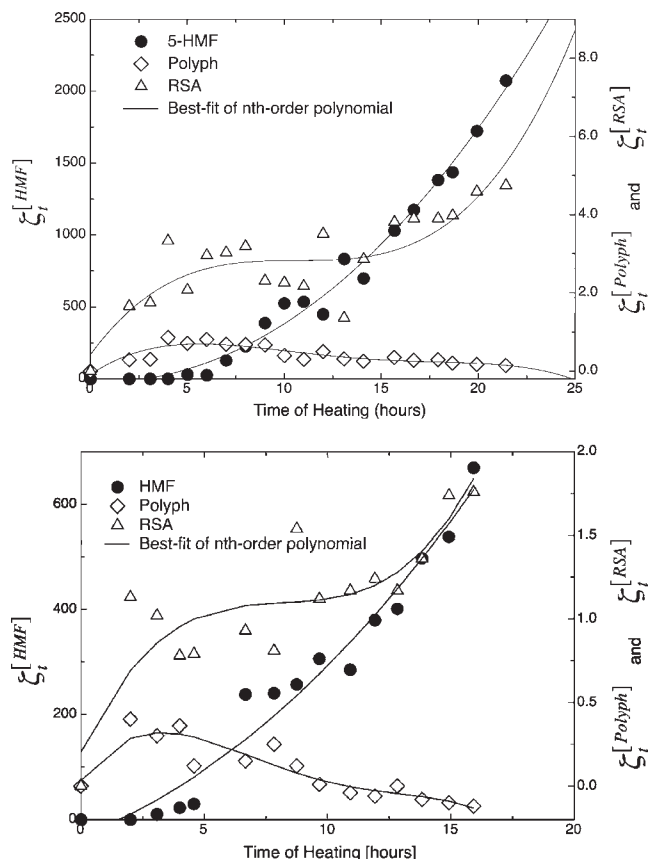*J. Agric. Food Chem.,* Vol. 58, No. 15, 2010   **8687**

Figure 5. Sugar degradation kinetics as described by the time of course for fractional conversion for 5-HMF, polyphenols equivalents, and RSA compounds. Fractional conversion level was treated as a quantitative index of the reaction extent and was calculated by taking into account volume lowering upon cooking.

products depend on the initial pH value (*27*). As a function of the pH value, lactic and acetic acids may be identified as products of 5-HMF degradation in acidic sugar solutions (*8*). Malic acid and fructose react in an aqueous solution at moderate temperatures in a Maillard-type reaction to form dark-colored end-products (*28*).

It is worth nothing that 5-HMF and radical-scavenging compounds converted positively to a greater extent when the volume of the grape must was reduced significantly by water vaporization: this is what occurred in the must from LRG. Differences in the starting composition of the two grape musts were too small to account for the observed conversions in both properties. All of these findings suggest that a complex role for water vaporization in the overall sugar conversion mechanisms can be hypothesized as the most probable pathways for sugar degradation in the grape musts used in our cooking experiments. Degradation pathways and the acid-catalyzed conversion of the enediolic form of reducing sugars (mainly fructose), leading to the stochiometric production of water, can be surmised. However, a contribution of the Maillard reaction can also be supposed, due to the complex behavior observed for both sugars and organic acids. On the other hand, the thermal degradation of numerous amino acids in grape must under cooking has recently been described (*29*). Conversely, caramelization mechanisms can be discounted as negligible, because the bulk temperature of the grape musts was never > 95 °C during the cooking.

As far as phenolic and radical-scavenging compounds are concerned, their fractional conversion was plotted against the time of cooking (reported in **Figure 5**). The former increased to a maximum level in the early stages of cooking and then decreased

continuously throughout the process; the latter showed positive conversion trends. An initial increase in antiradical activity, followed by a decrease, was observed for catechin solutions as well as in grape extracts (*30*). Qualitatively similar but quantitatively different trends were observed for the two batches of grape musts. Differences in total phenolic compounds throughout cooking were explained on the basis of the strong tendency of these compounds to suffer polymerization reactions. It is a widely known fact that henolic compounds can undergo polymerization, especially if favored by acidic pH and reduction in water content (*31*). In explaining the positive conversion of phenolic compounds, we hypothesize some rearrangements in their structure, at the early stages of cooking, which may enhance their ability to react with horseradish peroxidase/H_2O_2 under test conditions. It is known that that when the para-position of a phenol is already occupied, the reaction with horseradish peroxidase/H_2O_2 takes place to a great extent in the ortho-position, the new oxidation adduct being more stable. However, when the degree of polymerization exceeds a critical value, increased molecular complexity promotes a decrease in antioxidant capacity, probably because of steric hindrance, which reduces the availability of the phenolic rings for the horseradish peroxidase activity. An extended polymerization of natural phenolic compounds could explain their negative conversion at the advanced stages of cooking.

Differences in antiradical activity observed in the two investigated grape musts were explained on the basis of two main likely causes: (i) the strong tendency of the phenolic compounds to suffer polymerization reactions and (ii) the formation of new active compounds from sugar degradation. The increasing antiradical capacity during the early stages of cooking might be imputed to the more significant areas of charge delocalization on the polymeric structures arising from phenolic polymerization. It is widely accepted that the radical-scavenging capability of phenolic compounds is due to their hydrogen-donating ability: the greater the number of hydroxyl groups, the higher the possibility of free radical-scavenging activity. The availability of the hydroxyl groups depends closely on both their chemical structure and their spatial conformation, which can modify the reactivity of the molecules. Indeed, oxidative polymerization and oxidativermation of hydroxyl groups are widely known to be responsible for the enhancement of antiradical activity in catechin, the main polymeric phenols present in grape must. However, the extent of phenol polymerization is presumed to lower the radical-scavenging ability of such compounds, probably because of steric hindrance, which reduces the availability of the hydroxyl groups (*31*). The experimental evidence of the continuous increase of the radical-scavenging activity of the investigated grape musts can be attributed to other active compounds newly formed by sugar degradation. The latter hypothesis is supported by the vectorial relationships between RSA, 5-HMF, and phenolic compounds, as well as by other recent studies. These relationships show, for example, that high radical-scavenging activity could be linked with the presence of high molecular size melanoidins as already proved in grape must derivatives (*19*). The trend of the three properties suggested also that cause−effect relationships could be surmised between sugar melanoidins and in vitro radical-scavenging activity, as already proven in coffee and sweet wines (*33*).

The relative low fractional conversion levels calculated for both sugars (**Table 2**) indicates that they were not the limiting reactants in degradation reactions: the stoichiometry of acid-catalyzed degradation of glucose and fructose indicates that from 1 mol of sugar can maximally be obtained 1 mol of 5-HMF and 3 moles of water. In other words, sugar degradation was thermodynamically favored due to the high concentration of the reactants, but kinetically limited, with the result that a yield of only

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Table 2. Extent of Cooking Process and Extent of Cooking Effects on the Grape Must Properties[a]

		Must from SRG ($Vt/V0 = 0.586$)				Must from LRG ($Vt/V0 = 0.447$)			
		concn in fresh must, $I[0]$	concn in cooked must, $I(16)$	concn in cooked must, $I(16)^{b}$	fractional conversion,[c] $\zeta_{16}^{[I]} \times 100$ (%)	concn in fresh must, $I[0]$	concn in cooked must, $I(16)$	concn in cooked must, $I(16)^{b}$	fractional conversion,[c] $\zeta_{16}^{[I]} \times 100$ (%)
extensive properties (g/100 g)	DE	19.05	32.5	19.05	0	18.10	40.43	18.10	0
	RS	15.35	23.45	13.74	−10.5%	17.97	37.13	16.63	−7.4
	Brix	16.50	25.55	14.97	−9.3	18.90	40.30	18.04	−4.5
	D-Glu	7.38	12.14	7.12	−3.51	9.25	19.38	8.68	−6.21
	D-Fru	6.43	12.23	7.17	+11.5	9.40	19.27	8.63	−8.21
	Glu + Fru	13.81	24.37	14.29	+3.47	18.65	38.65	17.31	−7.21
	D-MaH	0.5	0.84	0.49	−2.01	0.4	0.8	0.36	−10.1
	D-LaH	0.0029	0.0035	0.00205	−29.3	0.0022	0.0084	0.0038	+72.7
	L-LaH	0.0019	0.0027	0.0016	−10.3	0.0021	0.0072	0.0032	+52.4
	AcH	0.014	0.0012	0.00075	−95.6	0.0019	0.0008	0.0003	−84.2
	TitAc	0.95	1.405	0.82	−13.7	0.69	0.8	0.36	−10.1
	5-HMF	0.00023	0.4335	0.2537	+110208	0.00062	0.9290	0.4159	+66980
	RSA	0.0041	0.03275	0.0192	+368.3	0.00838	0.04706	0.02107	+151.7
	polyph	0.00639	0.01380	0.00808	+26.4	0.00664	0.01190	0.0053	−20.2
bulk properties	pH	2.64	2.59	2.59	−1.19	2.75	2.63	2.63	−4.36
	a_w	0.992	0.966	0.966	−2.66	0.989	0.929	0.929	−6.46
	density	1.0710	1.1101	1.1101	+3.65	1.0818	1.1849	1.1849	+9.53

[a] Fractional conversion levels allow discrimination between the individual contributions of water vaporization from the overall cooking process, which is driven simultaneously by water vaporization and chemical reactions. [b] Concentration data were recalculated, taking into account the volume lowering ($V(t)/V(0)$), where $V(t)$ was the actual volume and $V(0)$ the volume of the fresh must (zero cooking time), assuming water vaporization to be the only stream leaving the must volume upon cooking. [c] $\zeta_{16}^{[I]}$ indicates the extent of chemical conversion of the compound (I) after 16 h of cooking for reactions in which it is involved as reactant, intermediate, or product. Fractional conversion level calculated for bulk properties refers to their change with respect to the initial value.

Table 3. Performance Criteria for Chemical Efficiency in Terms of Sugar to 5-HMF Conversion, [E_{HMF}] [a]

E_{HMF}^{b}	Must from SRG ($V(t)/V(0) = 0.586$)		Must from LRG ($V(t)/V(0) = 0.447$)	
nHMF/nΔ*[D-Glu]	(0.2535/126.11)/(0.26/180)	138.42%	(0.41528/126.11)/(0.57/180)	103.98%
nHMF/nΔ* [D-Fru]	(0.2535/126.11)/(−0.74/180)	−48.61%	(0.41528/126.11)/(0.77/180)	76.97%
nHMF/nΔ* [D-Glu + D-Fru]	(0.2535/126.11)/(−0.48/180)	−75.38%	(0.41528/126.11)/(1.34/180)	44.23%
nHMF/nΔ*Brix	(0.2535/126.11)/(1.53/180)	23.52%	(0.41528/126.11)/(0.86/180)	68.92%
nHMF/nΔ*RS	(0.2535/126.11)/(1.61/180)	22.36%	(0.41528/126.11)/(1.34/180)	44.23%

[a] E_{HMF} was evaluated after 16 h of cooking and calculated with respect to the moles (n) of reactants and products. [b] nΔ*, reacted amount of sugars.

5-HMF was obtained with respect to the initial sugars. Furthermore, due to the presence of other parallel reactions that may either reduce or enhance the final content of 5-HMF, different selectivity indices for sugar conversion in 5-HMF [E_{HMF}] and radical-scavenging compounds [E_{RSA}] were calculated after 16 h of cooking (**Tables 3** and **4**). In particular, E_{HMF} was expressed as a molar ratio between the 5-HMF and reacted sugars, whereas E_{RSA} was expressed as a mass ratio between the RSA and reacted sugars. Both fractional conversion and selectivity indices were proposed as performance criteria of the cooking process in terms of sugar degradation. As can be inferred from **Tables 3** and **4**, fractional conversion data indicated a major extent of sugar degradation in the musts from LRG, whereas selectivity data indicated a major selectivity of must from LRG in terms of 5-HMF and RSA compound formation.

From a chemical standpoint, both fresh and cooked grape musts are far from being in a state of equilibrium. Chemical potentials were thermodynamically permissive for sugar degradation in the fresh grape must: in fact, 5-HMF was found at 0.23 and 0.62 ppm in the fresh musts obtained from SRG and LRG, respectively. Cooking affected 5-HMF conversion differently in the two batches. In particular, the lag periods for 5-HMF accumulation were different between the two grape musts; that is, about 7 and 4 h in the SRG and LRG musts, respectively. After 16 h of cooking, the concentrations of 5-HMF were about 4500 and 1500 times the initial level (i.e., in the fresh must) for the two batches, respectively. Due to the higher concentration of 5-HMF with respect to the fresh

Table 4. Performance Criteria for Evaluating Chemical Efficiency in Terms of Sugar to RSA Conversion, [E_{RSA}][a]

E_{RSA}^{b}	Must from SRG ($V(t)/V(0) = 0.586$)		Must from LRG ($V(t)/V(0) = 0.447$)	
mRSA/mΔ* [D-Glu]	0.0151/0.26	5.81%	0.0127/0.57	2.23%
mRSA/mΔ* [D-Fru]	0.0151/-0.74	−2.04%	0.0127/0.77	1.65%
mRSA/mΔ*[D-Glu + D-Fru]	0.0151/-0.48	3.14%	0.0127/1.34	0.95%
mRSA/mΔ*Brix	0.0151/1.53	0.98%	0.0127/0.86	1.48%
mRSA/mΔ*RS	0.0151/1.61	0.94%	0.0127/1.34	0.95%

[a] E_{RSA} was evaluated after 16 h of cooking and calculated with respect to the mass (m) of reactants and products. [b] mΔ, reacted amount of sugars.

musts, cooked musts present much greater chemical potential in terms of sugar degradation ability. The greater the final concentration of 5-HMF at the end of cooking, the greater the increase expected in 5-HMF conversion ability. This is because 5-HMF's ability to act as polymer-building blocks is also widely recognized in sugar solutions (*32*), whereas complex polymerization reactions have been observed in cooked must during long-term storage (*25*).

Analysis of Sugar Degradation Kinetics. Kinetics of fractional conversion for 5-HMF and phenolic and radical-scavenging compounds was analyzed by fitting an nth-order polynomial to the experimental data; the rate of chemical conversion for 5-HMF (dζ[HMF]/dt), Polyhenols dζ[Polyph]/dt) and RSA compounds dζ[RSA]/dt) was then calculated by numerical derivation of the
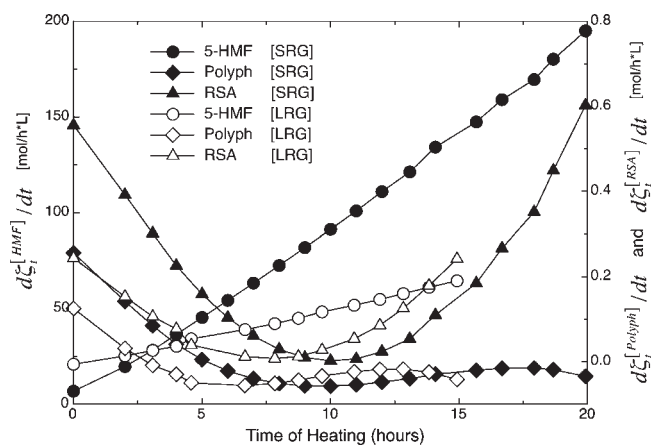
Figure 6. Rate analysis against the time of cooking. Fractional conversion in 5-HMF was used as a marker for sugar degradation at the earlier stages, whereas fractional conversion in the compounds with RSA was used as a marker for sugar degradation at the advanced stages. Different *n*th-order polynomial functions were fitted to the fractional conversion data and numerically derived to calculate the rate of fractional conversion.

best-fit equation. **Figure 6** shows the rate of these three chemical conversions against cooking time. It is evident that cooking accelerated the 5-HMF formation much more in the musts from SRG. With regard to the radical-scavenging compounds, the rate of conversion followed a complex behavior. First, it decreased to reach a minimum value; thereafter, it increased continuously throughout cooking: the minimum conversion rate in RSA for the musts from LRG was noted as having forward-shifted with respect to the cooking time, most probably because of the faster water vaporization (**Figure 2**). Our opinion is that phenolic compounds degraded at the first stage of cooking, next at the intermediate and advanced stages, and the sugar conversion leads to the formation of new compounds having RSA activity. On the basis of their different kinetics behaviors, fractional conversion of 5-HMF was proposed as a chemical marker for early-stage sugar degradation inside the grape must under cooking, whereas fractional conversion of RSA was proposed as a chemical marker for the advanced sugar degradation.

The conversion rate of 5-HMF and RSA compounds was analyzed against three bulk properties (pH, a_w, and Brix), with results recorded in **Figure 7**. Data showed that sugar degradation kinetics was much more sensitive to the bulk properties in must from SRG, as indicated by the higher 5-HMF and RSA conversion rates. In particular, the rate of 5-HMF formation changed with an inverse linear relationship with water activity and pH, whereas at the same time it increased, in line with a power law, with Brix. As far as the pH levels are concerned, although the initial pH values were very low (2.64 and 2.75 for fresh musts from SRG and LRG, respectively), they continued to decrease throughout cooking reaching, being, after 16 h, 2.59 and 2.63 in the two grape musts. Such variations are of more than marginal importance; in fact, a recent study demonstrated the key role played by minimal variations of pH on 5-HMF formation in a system simulating grape must (*15*).

All of these findings indicated that cooking speed increases the formation of 5-HMF and RSA according to the initial and actual amounts of free water and organic acids. Such behavior is more evident in the must from SRG: there, a greater volume lowering was observed during cooking.

A double effect of cooking can be hypothesized on the sugar degradation, especially at the advanced stages. It causes water vaporization and, therefore, direct loss of free water leaving the open vessel; simultaneously, solute concentration, glucose and fructose, in

particular, are presumed to contribute to the lowering of free water molecule concentration due to its high hygroscopic capacity. As a consequence, the proton-catalyzed step involved in acid-catalyzed sugar degradation is continuously shifted forward throughout cooking, with an increase in the amount of the degradation products. Our hypothesis is largely supported by results from the first study focused on the formation of 5-HMF in sugar solutions (*34*). The authors of the latter study observed that the formation of 5-HMF at fixed and limiting sugar concentrations was a function of water activity and to a lesser extent of initial sugar concentration and pH value. In particular, an inverse linear relationship was found between 5-HMF concentration and water activity varying from 0.779 to 0.889, whereas with respect to pH value, an inverse power relationship was found, varying from 2.9 to 3.2.

In conclusion, cooking experiments conducted in this work allowed us to infer that from an engineering standpoint, the vessel behaved as a nonisothermal batch reactor in which the volume of the grape must was lowered and its composition changed profoundly by chemical reactions. Through multivariate analysis of the experimental data, a small fraction of the must properties was selected and used as a marker for cooking extent (MCE): these properties included the relative changes of Brix, total titratable acids, acetic acid, malic acid, lactic acid (D and L), pH, water activity, 5-HMF, and phenolic and radical-scavenging compounds. Under our conditions, cooking was driven by two main engineering factors: (i) water vaporization resulting mainly in a lowering of the volume and, therefore, of the solute concentration and (ii) the sugar degradation reactions. Cooking allowed opposite conversion of both sugars and acids with different kinetics between the musts from short- and long-ripened grapes. The acid-catalyzed conversions of the enediolic form of both fructose and glucose were hypothesized as the most probable pathways for sugar degradation in the grape musts under investigation. However, a contribution of Maillard reaction to the sugar degradation was also surmised. Additionally, other parallel reversible reactions, involving both the sugars and malic acid, were also hypothesized. It was recognized many years ago the role of HMF as an intermediate compound in sugar degradation reactions; that is, its concentration first increases and then decreases during a sugar conversion experiment. However, all studies focused on HMF formation were based on a closed system, where none of the reactants or products were able to leave the system themselves. The early, intermediate, and advanced stages of sugar conversion analyzed in this work are referable strictly to the grape musts under our cooking conditions. Specifically, the conversion of the reducing sugars occurred in an open system (atmospheric vessel), where water vaporization takes place extensively over the time and where the water played a direct role in sugar conversion reactions, including HMF formation and RSA changes. Under these conditions, in fact, it was hypothesized that the equilibrium dehydration steps of sugar conversion via acid-catalyzed mechanism, releasing 3 mol of water for each sugar mole, is continuously shifted forward throughout cooking. As suggested by the experimental data, HMF forms and degrades continuously, but water elimination was accelerated by the water vaporization process so that HMF increased monotonically throughout cooking. This behavior does not confute the role of HMF as intermediate compound in the sugar degradation reactions, but its fractional conversion can be advantageously used to describe the extent of the degradation reactions in grape must at the early stage of cooking.

The data suggested that the continuous degradation of natural phenolic compounds, more likely occurring through a polymerization mechanism, was simultaneous with the sugar degradation. Furthermore, new and different products with radical-scavenging capacity arise at the advanced stages of sugar degradation. Due to
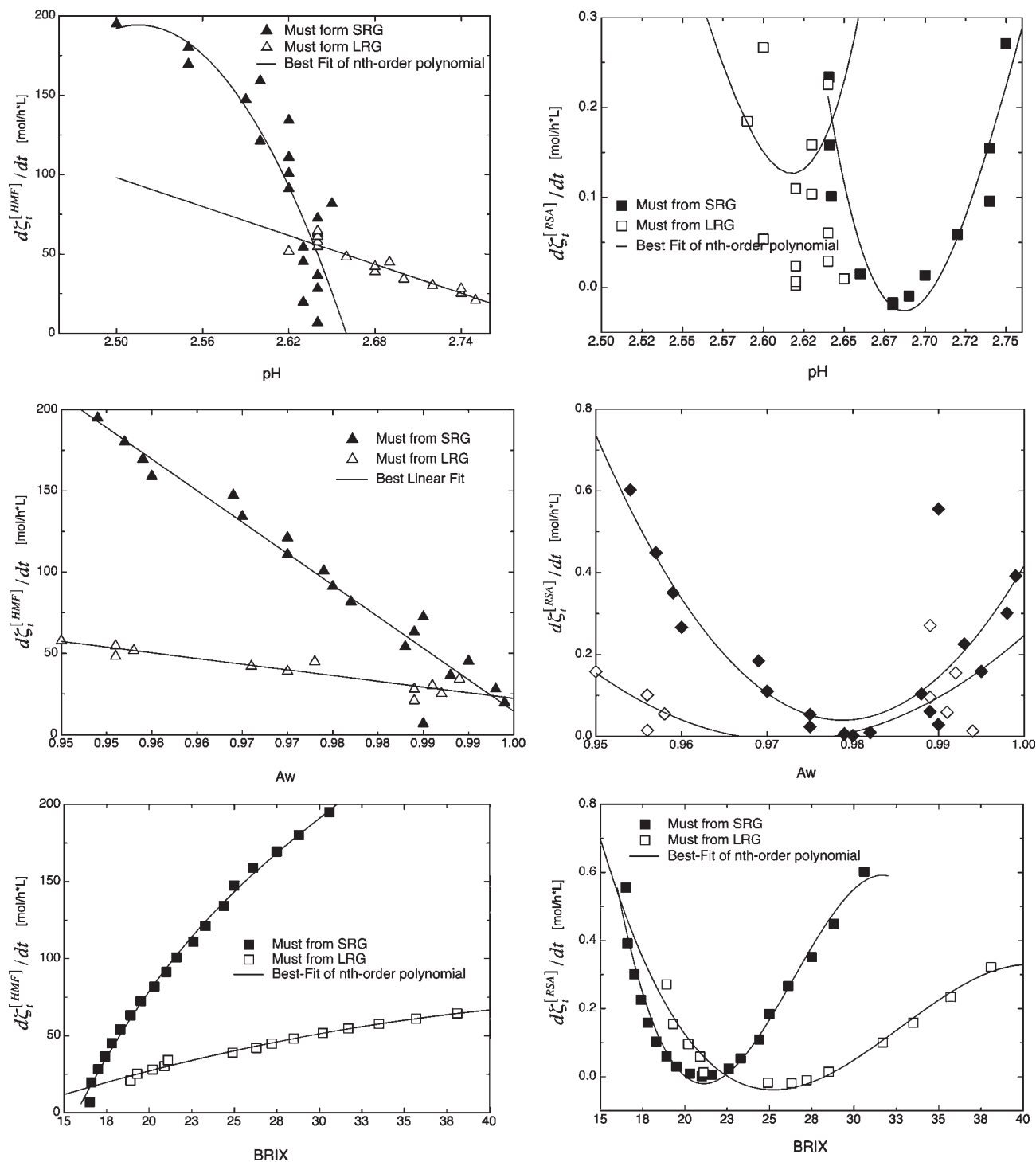
Figure 7. Influence of the initial and actual levels of three bulk properties such as pH, water activity, and Brix as fixed by cooking on 5-HMF and RSA conversion rate.

their different kinetic behaviors throughout cooking, fractional conversion of 5-HMF was proposed as a chemical marker for early-stage sugar degradation, whereas fractional conversion of RSA was proposed as a chemical marker for advanced-stage sugar degradation. Finally, different selectivity indices were proposed as performance criteria of the cooking process in relation to sugar degradation.

Results from this preliminary work will be used to design correctly the solute mass and heat balances aiming to account for chemical conversion involving sugar, 5-HMF, and radical-scavenging compound formation. In this way, the extent to which

a grape must's composition affects the final chemical and physical properties at given conditions for cooking, as well as the cooking conditions required to ensure desired final properties in grape juice composition, will be addressed, with the aim of reducing the final content of 5-HMF.

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