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Changes in the Chemical Composition of Reduced Cooked Musts during the Heating Process

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Cooked must is the starting raw material in Aceto Balsamico Tradizionale di Modena (ABTM) production, and the cooking process is a crucial step to ensure quality and safety standards. In particular, the furfural content has to be strictly monitored. In this study, we followed, directly at the production plant, nine cooking processes, differing for grape type and process conditions in order to monitor the actual variability in cooked must reduction. 5-Hydroxymethylfurfural (5-HMF) and furfural contents were determined by high performance liquid chromatography (HPLC) technique and glucose, fructose, and tartaric and malic acids by gas chromatography (GC) technique. Furthermore, some bulk properties, such as the water content, refractive index, density, and total acidity, were also measured. The obtained results show that the product undergoes, in the worse operating conditions, remarkable degradation, leading to extremely high levels of furfurals (5-HMF and furfural), corresponding to a water content lower than 40%. On the basis of these results, a first draft of an optimal heating protocol may be suggested in order to guarantee the safety and quality of the final product.

KEYWORDS: Cooked must; 5-HMF; furfural; glucose; fructose; organic acids

INTRODUCTION

Quality and safety assurance are necessary requisites for foodstuff production. In particular, recently health emergencies have also made the consumers aware of the utmost importance of having declared information about the origin and processing of foods. The European Union has recently introduced regulations to guarantee and protect against frauds the traditional food production by codifying the qualitative characteristics relative to typical foodstuff products, together with their transformation processes. In other words, it has introduced a system of labeling in order to simply and immediately reassume the peculiarities of the labeled food. Although the denomination and declared characteristics of the food are strictly related, on the contrary there is no absolute and objective equivalence between quality and safety of the product.

Institutional actions aimed at improving foodstuff safety and quality have never been stopped, but the safety policy has been recently reviewed, as a consequence, for example, of the panic generated in the 90s around the so-called mad cow syndrome, or food contaminated by dioxin, or adulterated wines and olive oils. Even if zero risk is not a possible objective, the European Union tries to contain it by adopting modern norms based on advanced scientific tools.

Control systems to guarantee foodstuff security are in action in all countries belonging to the EU and have to comply community rules, but at the same time leave room for traditional food products and local specialties, allowing for some diversity. Substantially, the EU is an active promoter/ committee of diversity and quality, it protects typical and traditional products from treacherous copies, and promotes biological agriculture. The aim is not to limit innovation or to homogenize food products available on the European market; instead, it is to fix fundamental norms of safety both on communitarian (*1*) and international (*2*) contexts in order to develop and implement quality and excellence, and at the same time guarantee a high degree of food safety while maintaining the peculiarities of each food production. Among the different issues with regard to foods transformation processes, food safety, and hygiene, the problem of furfural formation is nowadays one of the main topics of interest for many researchers (*3–10*), foods safety authorities, and international organizations around the world, such as EFSA (European Food Safety Authority) (*11, 12*), FDA (Food and Drug Administration) (*13*), and FAO (Food and Agriculture Organization) (*2*). Furfurals are organic compounds with high volatility and lipophilicity, used in various chemical-manufacturing industries. Furfural and 5-HMF can be formed in foods that undergo heat treatment including home cooking. The

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presence of furfurals in foods as volatile flavors has been known for many years, and a basic formation pathway through carbohydrate thermal decomposition has been postulated (*10, 14*). Furfurals occur in a variety of foods such as coffee (*15*) and canned and jarred foods (*5*) including baby food (*4, 6*). In particular, it has been demonstrated that, among the chemical processes that cause furanic species formation, such as 5-hydroxymethylfurfural, furan, and 2-acethyl-3-hydroxyfuran, the drastic/prolonged heating of concentrated sugar solutions, such as grape must, is one of the main reactive pathways (*4–10*). Moreover, the interest toward these species is confirmed and supported by the initiatives promoted by European Commission. The European Food Safety Authority (EFSA) established for furfural an acceptable daily intake ADI $= 0.5\ mg \cdot Kg^{1-}$ of body weight (*11, 12*), and recently, the European Community has promised to finance projects and advance research focused on the investigation of furfural content in food (*16*). Therefore, in this context, the present work is a first attempt to supply, on an analytical basis, information about the critical steps in the production of a traditional food in order to adopt preventive measures at the early production stage and improve the quality of the final product. In order to accomplish to these tasks, on one side, an extensive analytical investigation of the industrial cooked must production was at first planned to gain more experimental data relative to the monitoring of the real condition for the process under study, and in the end, some operative recommendations were proposed to the producers as a possible solution of the problem.

In particular, the work is part of a collaborative project involving producers, surveillance agencies, and representation consortia, aimed at obtaining new cognitive and operative tools to control the evolution of chemical and physical characteristic of the different grape juices varieties during the cooking process and the quality of the finally obtained cooked must, which is used as raw material in Aceto Balsamico Tradizionale di Modena (ABTM) production. The main objective was to optimize the grape must cooking process in order to obtain cooked musts characterized by a low content of furfurals. In fact, it is well known that these chemical species are toxic not only for yeasts and bacteria (*17, 18*), which carry out alcoholic fermentation and acetic bio-oxidation reactions during vinegar production, but also for the human organism (*11, 12*).

In the literature, some interesting preliminary investigations on the same topics as well on the same food matrix (*19, 20*) are present. In particular, these authors have studied the correlation of glucose/fructose ratio, furfural compound production, and time of the must cooking process.

However, they concern a limited set of samples taken from one producer of the local family tradition. On the contrary, this work addresses the commercial production of the ABTM raw material by representative sampling directly of the wine cellars. In particular, the relevant aspects are (i) the number of the investigated heating procedures, i.e., nine heating cooking processes; (ii) the number of samples taken at different times for each heating procedure; (iii) the representativeness of data sampling with respect to the whole reduced cooked must production of the district of Modena; and (iv) the analysis of the real case conditions adopted by the factory producers, which were different for the various heating processes.

Therefore, the aims of this work are the extensive characterization of the raw materials used in ABTM production and the study on the influence of the technological apparatus and processes on the composition and the transformation. To this aim, nine cooking processes were directly sampled at the production plant involving different grape types and production apparatus. In a prior publication (*20*), fructose, glucose, furfural, and 5-HMF concentrations were determined in must samples in order to study the changes in composition of cooked must as a function of cooking time, different grape varieties, and applied cooking technologies. In fact, the effect of the cooking apparatus was studied by varying the geometry of the pans used for must cooking, i.e., with a circular section equipped by an automatic stirrer or with a rectangular section not provided by any stirring equipment. However, now, the state of art of must production is monitored since the cooking processes were directly followed within the traditional procedures, without making correction or changes in the cooking strategy usually employed by the producer (*21, 22*).

As quality indicator of cooked must product during its preparation, 5-HMF and furfural were considered. Furthermore, fructose and glucose, which undergo concentration effects and degradation phenomena, involving furfural formation, as well as tartaric and malic acids were also monitored. Moreover, water content was measured since its loss represents an important and crucial physical phenomenon during the heating process due to the dehydration (oxidative) reaction of the reducing sugars, mainly fructose and glucose, that lead to 5-HMF and furfural formation. Finally, refractive index, density, and total acidity variables were also measured in order to characterize the bulk of the whole system.

The obtained results highlighted the most critical cooking conditions and constitute a useful basis to define an optimal cooking procedure.

MATERIALS AND METHODS

Chemicals. Deionised water for samples, standards, and eluent preparation was obtained by a Millipore Milli Q185 Plus system (Millipore, Bedford, MA).

Sodium hydroxide (0.1 M), used in the titration for total acidity determination, was provided by Fluka. The concentration of the 0.1 M NaOH was checked daily by potassium phthalate, a primary reference material for alkalimetry, certified by EMPA (Materials Science and Technology Research Institute, Federal Institutes of Technology, ETH, Zurich) and BAM (Federal Institute for Materials Research and Testing, Germany), with $a \geq 99.5\%$ purity supplied by Fluka, Milan, Italy.

The water content in the cooked musts were evaluated by Karl Fischer titration. Hydra-point composite 5 for Karl Fischer titration was supplied by J.T. Baker. Sodium tartrate dihydrate with $a \geq 99.5\%$ purity, supplied by Fluka, Milan, Italy, was used as the standard for Karl Fischer Titration.

Solid phase extraction cartridges, C18 500 mg, were supplied by Supelco and conditioned by a solution prepared by using Methanol with $a \geq 99.8\%$ purity supplied by Riedel-deHaen. The same methanol was used as solvent in the KF titration.

Standards of organic acids, tartaric and malic acids, and sugars, glucose and fructose, were supplied by Fluka with a purity greater than 99.5%. Phenyl-β-D-glucopyranoside (internal standard for GC analysis) and *p*-hydroxybenzoic ccids (internal standard for GC analysis) with purity greater than 99% were purchased from Sigma Aldrich, (Milan Italy). The oximant reagent, hydroxylamine hydrochloride, with purity greater than 99%, was supplied by Carlo Erba. Pyridine Ultrapur, used to prepare oximant and standard solutions, was provided by Fluka. Silylation reagent (BSTFA plus 1% TMCS) was provided by Supelco.

5-HMF and furfural with purity greater than 99% were supplied by Sigma Aldrich, (Milan Italy). TEG (triethylenglycol), used for the preparation of the furfural standard solution, was provided by Fluka. Suprapur 96% H_2SO_4, supplied by Merck, and HPLC grade CH_3CN, supplied by LABORATORY-SCAN, were used to prepare the eluent solution for HPLC analysis.

Equipments and Instrumental Settings. All samples and standards were prepared by weight by using a Mettler AE 200 (Metalchimica, Milan, Italy) analytical balance with a sensitivity of ± 0.1 mg.

During sample preparation for GC and HPLC analysis, samples were passed through SPE-C18 cartridges by using a Manifold system from Alltech equipped by a KNF Labport vacuum pump. Refractive index values were measured by a GPRX 11-37 limit angle refractometer, maintained at constant temperature (20 °C) by a HAAKE F3 thermostat with 0.01 °C temperature sensitivity.

During the cooking process, the temperature was measured by a probe supplied by Tersid: model 505 as an indicator equipped with a Pt100-3 as the thermoresistance probe. The amount of water in the samples was determined by a Mettler DL 18 model KF titrator. Total acidity of the samples was determined by pH-metric titration. The pH was measured by a Denver Instrument 215 model digital pH-meter equipped by a combined glass electrode (N42 BNC type, supplied by Schott Gerate, Milan, Italy).

Gas chromatographic determinations of organic acids and sugars were performed by a Varian 3400 GC provided with a flame ionization detector (FID). A nonpolar capillary column (Alltech AT-5 length 60 m, internal diameter 0.25 mm, film thickness 0.20 μm) was used. Helium was used as carrier gas (linear velocity 30 $cm \cdot s^{-1}$ evaluated at 150 °C). The split injection mode was used with a split rate 1:30. The FID and injector temperature were set at 310 and 280 °C, respectively. The column starting temperature was 80 °C, and it was increased at 4 $°C \cdot min^{-1}$ to 210 °C and then programmed to 250 at 2 $°C \cdot min^{-1}$. The temperature was finally increased to 280 at 4 $°C \cdot min^{-1}$ and held for 10 min.

High performance liquid chromatography determination of furfurals were performed by a Beckman model System Gold apparatus equipped with a single piston pump model 116, injection valve model 210A, 20 μL sample loop, and a Diode Array Detector model 168. A Hydrogenionic column (BioRad Ion Exclusion HPX-87H Aminex, 9 μm particle size, 300 mm length, 7.8 mm internal diameter) was used for the chromatographic separation of the solutes. The column was thermostatted at (65.0 ± 0.1) °C to obtain the best resolution conditions. Furfurals were quantified at $\lambda = 275$ nm. Mobile phase composition was 90% 0.005 N H_2SO_4 and 10% CH_3CN. Isocratic technique with a 0.6 $mL \cdot min^{-1}$ flux for a 45 min duration was performed for HPLC separation. Data were collected and analyzed using System Gold software 3.1 version.

Sampling. The ABTM making protocol prescribes that the cooking of must has to be performed in uncovered pans heated with direct fire (*21, 22*). However, cooked must production is somehow subjective, depending as well on the tradition of the single producer and is not codified in detail by the registered traditional protocol. For these reasons, in order to obtain a representative sample set, a collaboration with local cooked must producers has been established. In general, within cooked must production two different realities exist: (i) local producers of family tradition who perform cooked must production for their own ABTM production (personal consumption) and therefore according to their own tastes but always under the respect of the traditional protocol; (ii) commercial producers who operate in the context of wine cellars, always according to the traditional protocol specifications, and then sell the cooked must to different purchasers. Both kinds of production contexts were taken under consideration, but according to the aims of the present investigation, the second reality was mainly investigated, allowing a more representative sampling since it represents a significant percentage of cooked must production dedicated to ABTM making. Moreover, this also allowed the testing of the performance of different production apparatus and practices, which while always accomplishing the traditional heating method, i.e., direct fire heating in uncovered and cylindrical pans, introduce some differences in the cooking process. In particular, nine cooking processes performed by three different wine cellars have been monitored: grapes, of different varieties, were obtained from the 2006 harvest and processed in the period September−October, 2006. Must samples were taken regularly during the entire heating process, from the crude to the final product, hereafter named reduced cooked must.

Table 1 summarizes the must samples available for each cooking process (labeled C1−C9), grape variety (white or red), and the different wine cellar producers (kept anonymous and simply labeled as A, B, and C). Furthermore, details about the conduction of the heating process performed by different producers are also reported. The total number of collected samples is 122.

Producer A: Four Cooking Processes Sampled, Namely, C1, C2, C3, and C4. The white grape variety used is *Trebbiano Montanaro*. Grape was soft pressed to obtain the crude juice, and then the liquid was kept in the tank and maintained at 0 °C until its use. Four cooking processes have been contextually followed; each cooking process was performed in food grade stainless steel pans characterized by a circular section (2000 L capacity), equipped with an automatic stirring device consisting of four undulate shovels, which also improve water evaporation during the overall heating process. All the pans were filled up at the same time with the crude must after filtration. Stirring and thermal heating were immediately activated. When the liquid volume had decreased at approximately 1/3 of its initial value (approximately after 20 h), the stirrer was stopped, and the pan was refilled with the same initial crude must (conserved, in the tank, at $T = 0$ °C), maintaining the heating. The stirrer was turned on again after about 2 h, and no operation was carried out until the end of the heating process, which took a total of 43 h. In the cooking process labeled C1, an unexpected event happened between the 8th and the 20th hour of heating (during the first night). In particular, the heating was stopped because the boiler was accidentally turned off. As a consequence, a limited volume decrease took place; thus, refilling was not necessary. In summary, for each of the 4 cooking processes a must sample was collected with a 2 to 6 h time interval from the beginning until the end of the entire heating process, except for the time interval of the 8th and the 20th hour of heating, corresponding to the first night, where sampling did not occur because of operator unavailability (see **Table 1**).

Producer B: One Cooking Process Sampled, Namely, C5. Lambrusco Grasparossa is the red variety of grape used. Must was cooked in a food grade stainless steel cylindrical pan of 600 L capacity, characterized by a circular section equipped with the same type of automatic stirrer device described for the previous producer. Grape was soft pressed to obtain the crude juice, and then the pan was filled up with the unfiltered product. Heating was started immediately, while the stirrer was activated after some hours in order to let any deposit to settle down. Once activated, the stirrer system was kept on until the end of the heating process, corresponding to a total of 20 h. A must sample was collected each hour at regular time intervals (see **Table 1**).

Producer C: Four Cooking Processes Sampled, Namely, C6, C7, C8, and C9. Must samples were obtained from a mixture of red grape named *Lambrusco DOC*. Four cooking processes have been followed on different days (the same day for cooking processes C6−C7 and for cooking processes C8−C9) using the same crude must obtained by soft pressing of the grape and then stocked in a tank at 0 °C. Must heating was performed in 5 food grade stainless steel cylindrical pans, as follows: 2 identical pans of 600 L capacity, equipped with the automatic stirrer device and 3 identical pans, of 200 L capacity. Only the cooking processes conducted in the larger pans were sampled.

C6 and C7. All 5 pans were filled up at the same time with the grape juice. The stirrer system was activated after 4 h from the beginning of the heating. After about 6 h, when the liquid had decreased by approximately 1/3 of its initial value, a first refilling was executed as follows: the must, partially cooked, contained in one of the small pans was entirely used in order to refill the two large pans. (A representative sample from the small pan immediately before refilling and a representative sample from each of the two large pans immediately before and after refilling have also been collected.) After approximately 3 h, a second refilling procedure was performed as previously described by using the must, partially cooked, contained in one of the other small pans. A third refilling was also performed about after 13.5 h. From this moment, the heating process proceeded until the morning of the day after for a total duration of 22 h. In summary, a must sample was collected every 2 h at regular time intervals corresponding to the refilling steps (**Table 1**). No samples were collected during the night.

C8 and C9. The must used as raw material is the same processed during cooking processes C6 and C7; this is the case in the last aliquot contained in the tank. The raw must available was sufficient to fill up

Table 1. Summary of Available Samples for Each Grape Variety, Producer, and Cooking Process[a]

Grape	White				Red				
Producer	A				B	C			
Cooking process	C1	C2	C3	C4	C5	C6	C7	C8	C9
Time interval of sampling (hours of heating)	time 0 (the same crude must)				time 0	time 0 (the same crude must; at the low of the tank)		time 0 (the same crude must; at the bottom of the tank)	
	2	2	2	2	1	2	-	2	2
	4	4	4	4	2	4	4	4	4
	6	6	6	6	3	6	6	6	6
	8	8	8	8	4	1st refilling (sampling also from small pan, labelled R1)		refilling (sampling also from both small pan , labelled R1 and R2)	
	first night shift (no staff at work in order to collect samples; boiler turning off for pan C1)				5				
					6	6,5	6,5		
	20	20	20	20	7	8	8		
	-	refilling			8	8,75	8,75		
	22	-	22	-	9	2nd refilling (sampling also from small pan, labelled R2)		6,75	6,75
	23	23	23	23	10			8	-
	24	-	24	-	11	9	9	10	10
	26	26	26	26	12	10	10	12	12
	28	-	28	-	13	12	12	14	14
	30	30	30	30	14	13,5	13,5	night shift (no staff at work in order to collect samples)	
	32	-	32	-	15	3rd refilling (sampling also from small pan, labelled R3)			
	35	35	35	35	16				
	38	-	38	-	18	14	14		
	41	41	41	41	19	night shift			
	43	43	43	43	20	22	22	22	22
N°of samples	17	12	17	12	20	16	15	12	11
Total number of available samples = 122									

[a] Samples are arranged as a function of heating time. Some information/specification/notation relative to the peculiar strategy used in the heating process performance is given.

the two large pans and only two small pans (one entirely and one approximately at 2/3 volume). Since must was limpid, the stirrer was immediately activated. After approximately 6 h of heating, refilling was performed by taking the must from both of the small pans. In summary, a must sample was collected every 2 h at regular time intervals and during the refilling procedure. No sampling occurred during the night (**Table 1**).

For all monitored cooking processes, several aliquots of liquid were sampled at different positions and depth of the pans in order to obtain a representative bulk sample of 200 mL. Samples were stored at 0 °C to minimize any fermentation phenomena. Only temperature was determined at the moment of sample collection. The other physical and chemical measures were determined as quickly as possible, taking about one month to accomplish all analyses. Thus, before each determination the sample was reconditioned to room temperature and filtered on a cotton wool filter in order to eliminate suspended particles.

Determination of Bulk Parameters. *Temperature.* During each cooking process, the temperature was monitored either with the temperature sensor part of the heating apparatus or by a temperature probe immersed in the pan corresponding to the time of sample collection. Instrumental sensitivities differ depending on the apparatus at the producer's disposal. Pans used by producers A and C have the same capacity and geometry and were provided with a temperature sensor with an associated instrumental error of ± 1 °C; the temperature sensor used by producer B had an associated instrumental error of ± 2 °C. The temperature values of samples collected during cooking processes C6 and C7 have been obtained by using a temperature probe with a working range −80−600 °C and a ±0.1 °C associated instrumental error. (The different errors associated with temperature values are reported in notation (a) in **Tables A1**, **A2**, and **A3** in Supporting Information.)

Refractive Index, n_D, and Density, d. Each sample was analyzed once because of the good reproducibility shown by both methods, as tested by replicating 3 times the analysis of 3 different samples of one of the studied cooking process, namely, samples C5, 1 h; C5, 10 h; and C5, 20 h (associated errors are reported in notation (b) and (c) in **Tables A1**, **A2**, and **A3** (Supporting Information)).

Total Acidity. The total acidity for cooked must almost corresponds to the not-volatile acidity, because volatile species, such as acetic and lactic acids, if eventually present in the crude must, are rapidly evaporated because of the temperature values reached during heating (not lower than 80 °C). Although acetic acid is absent, it is custom to express the total acidity value, as for balsamic vinegar, as $g_{\text{Acetic Acid}}$ in $100g_{\text{sample}}$. Total acidity is determined by titration. A pH versus mL_{NaOH} curve was obtained for each sample, having verified the good reproducibility of the method by replicating 3 times the analysis of the 3 control samples (C5, 1 h; C5, 10 h; and C5, 20 h). Total acidity value, for each sample, was obtained by averaging the values calculated from the curve on the basis of the first, second derivative, and tangent methods. The associated error values reported in **Tables A1**, **A2**, and **A3** (Supporting Information) correspond to the standard deviation associated with the values obtained by the different methods.

Water Content, H_2O. Water content was determined by the Karl Fisher Titration method. The standardization of Karl Fischer Titration solution was performed by repeating the analysis 5 times, first by using a standard of sodium tartrate dihydrate and then monitoring daily during the measurement period. Each sample was analyzed twice, and the error associated with the determination as reported in **Tables A1**, **A2**, and **A3** (Supporting Information) has been calculated by error propagation considering both the standard deviation value obtained for repetition with KF reagent and the standard deviation value obtained by sample replication.

HPLC Determination of 5-HMF and Furfural. The analytical method applied is fully described in ref *20*. Samples were diluted roughly three times by weight with deionized water. Successively, 5 mL of the diluted solution were passed through a C18 SPE cartridge, previously activated with 2 mL of a 9:1 water/methanol (H_2O/CH_3OH) mixture. This last step was performed to eliminate phenolic compounds that may interfere with the analytical determination (*23*). The eluted solution was also diluted twice (if the initial must sample was cooked less than 12 h) or 5 times (if the initial must sample was cooked more than 12 h) and then analyzed by liquid chromatography. Each analytical sample was prepared twice and injected twice for the HPLC determination.

Individual analytes in the samples were identified by comparison of their retention times with those of standard compounds prepared in the same way. In order to perform a quantitative analysis, the optimal calibration curves, in terms of calibration range to minimize the associated uncertainty (*24*), were calculated both for 5-HMF and for furfural by preparing a multiple standard mother solution in triethylene glycol (TEG), then diluting in water at different concentrations to cover the analytical range (in particular, from 10 to 700 $mg \cdot kg^{-1}$ for 5-HMF and from 0.2 to 1.5 mg kg^{-1} for furfural). The area values obtained from the signal integration on the chromatogram were interpolated on the corresponding standard calibration curve in order to obtain the corresponding concentration values.

Periodic monitoring of the instrumental efficiency, i.e., of the reproducibility and the repeatability of the experimental and instrumental conditions over time, was performed by frequently preparing and injecting one of the standard solutions (in particular, 350 $mg \cdot kg^{1-}$ for 5-HMF and 0.85 $mg \cdot kg^{1-}$ for furfural).

GC Determination of Sugars and Organic Acids. The analytical method applied is fully described in ref *25*. The analytical method allows the simultaneous quantification of organic acids and sugars in ABTM samples, a matrix that closely resembles cooked must.

Sample were prepared as reported in the reference (after dilution and filtration on SPE cartridge, the solution was evaporated and the sugars converted to their oxime derivatives by hydroxylamine hydrochloride as oximant reagent; the oximes and the acids were then silylated), except for the dilution of the sample: after filtration through theC18 SPE cartridge. The collected eluate was not diluted to a 25 mL final volume but to a 10 mL final volume, depending on the lower content of sugars and organic acids in must compared to ABTM.

Each sample was prepared once and injected twice. Quantification was performed as reported in literature by means of the internal standard method and the calculation of response factor, K_i, by repeated injection of multiple standard solutions. Associated errors values were calculated as described in the cited paper, and literature data were used as recovery values.

Data Analysis. Chemometric techniques are used here in order to obtain a better graphical representation, rationalization, and a suitable interpretation of the data derived by the various analytical techniques. Because of the impossibility of overlapping samples belonging to different cooking processes because total duration and time interval are not the same for all the studied cooking processes, a three-way analysis was not applicable; however, a standard two-way organization of data appeared to be a useful approach considering that the number of samples is not prohibitive. In order to better evaluate the evolving trend with cooking time within each cooking process, the data were first column mean centered considering each cooking process separately (in this way for each considered variable, each sample is anchored to the average of its cooking process instead of the total average over all samples and producers) and subsequently arranged in a bidimensional matrix of dimensions 132 × 11 (samples at different time for the different cooking processes on the rows × measured variables on the columns). Then, before principal component analysis (PCA), autoscaling was applied.

PCA analysis was carried out by using the PLS Toolbox 4.0 for MATLAB (distributed by eigenvector Research Incorporated, WA, USA).

RESULTS AND DISCUSSION

Table A1 (Supporting Information) reports temperature (T/°C), refractive index (n_D), density (d/$g \cdot cm^{-3}$), total acidity ($g_{acetic\ acid}$ in $100g_{sample}$), water content (H_2O/%), 5-HMF, furfural, glucose, fructose, tartaric acid, and malic acid ($mg \cdot kg^{-1}$) values for cooking processes C1, C2, C3, and C4 performed simultaneously by producer A. **Table A2** (Supporting Information) reports the same parameters for cooking process C5 from producer B, and finally, **Table A3** (Supporting Information) reports the same parameters for cooking processes C6, C7, C8, and C9 performed by producer C. As regards to the different composition of the crude juices obtained from the various grape varieties, it is important to emphasize that besides grape variety, the weather and ground characteristics strongly affect the quality of the grapes during the harvest period with respect to sugars and organic acid content. The main differences regarding organic acid content is that it is generally higher in red than in white grapes and depends on the degree of grape maturation. The crude juice used in C5 shows a very low acidic concentration imputable to a late grape-harvesting, i.e., complete maturation of the grape.

For all of the studied cooking processes, the cooking temperature, after reaching the steady state of the heating conditions, was maintained roughly constant until the end of the process, depending on the precision of the control device. It is noticeable that C1, C2, and C3 cooking processes absolutely present the highest mean temperature values, >90 °C, instead of around 85 °C for the C4 and C5 cooking processes, about 80 °C for C6, C7, and C8, and finally, about 75 °C for C9. High temperature values could constitute a serious risk of obtaining a higher concentration of furfural compounds during cooking process, especially when this condition is accompanied by a low water content. Accordingly, the C2, C3, and C4 final products show the highest 5-HMF and furfural contents. It is worth noticing that the degree of furfural formation is directly proportional to the degree of the loss of water. Also, C1 was maintained at high temperature values, but in its case, the loss of water was not so evident causing an uncontrolled furfural production.

Other bulk parameters, i.e., n_D, d, and total acidity, are inversely related to water content; consequently, the highest values are registered for the final products obtained by cooking processes characterized by the strongest losses in water.

An evidence related to the very low water content in C2−C4 series is that final sugar concentration ranges from 720 to 800 $g \cdot kg^{-1}$, which represents a value very far from the one showed by the cooked must usually used for ABTM production. The compositional characteristics of these final products are certainly prohibitive with regard to their use as raw starting material for ABTM production, unless they undergo a dilution step.

For the sake of brevity, only one cooking process is discussed in detail by analyzing the trends of each single variable, leaving to discussion of PCA results the comparison among the various cooking processes. In particular, cooking process C4 was chosen because it is more representative of what could be on average a rather standard cooking process in a wine cellar context. However, C1 is clearly anomalous because of the inconvenience that occurred during the first night, while C2 and C3 might be considered anomalous because of the extreme conditions of the cooking process; the C6−C9 trends appear more peculiar because of the complicated refilling procedure performed.

Figures 1 and **2** show the trend of the refractive index, density, water content, total acidity, and temperature for C4 samples as a function of the number of hours of cooking (Time,

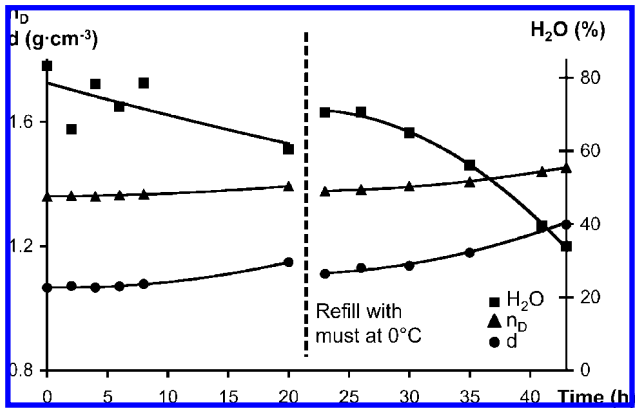

Figure 1. Trend of refractive index (n_D -▲-) and density (d -●-) are on the left axis scale, and water content (H_2O -■-) is on the right axis scale, for cooking process C4 samples as function of the cooking time (Time, h: hours). The dotted line refers to the refill procedure with crude must at 0 °C.

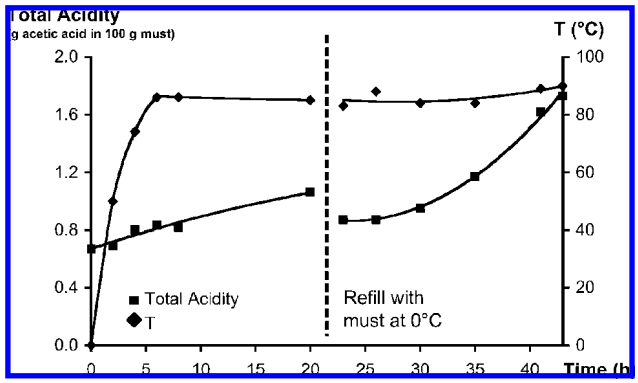

Figure 2. Trend of total acidity (-■-) and heating temperature (-◆-) for cooking process C4 samples as function of cooking time (Time, h: hours). The dotted line refers to the refill procedure with crude must at 0 °C.

on abscissa). The curves are drawn only to emphasize the general trend of the variable during the entire cooking process and therefore are not intended to fit the data, i.e., they should not be seen as regression/interpolation curves.

From **Figure 1**, it is possible to observe that initially the refractive index and density values increase during cooking following almost a linear trend due to the concentration effect inside the matrix as consequence of water evaporation. These trends break after 20 h because of the refilling with crude must at 0 °C, then increase again until reaching the final refractive index and density values. The total loss of water is very high, going from 83% to 34%. As regards the total acidity data, a trend comparable to those of the d and n_D is appreciated; in fact, a linear increase of the acidity is observed until the moment of refilling, then the variable increases until reaching the final value. This trend is probably due to a concentration effect of the acidic species in solution since during must cooking, specific reactions, involving organic acid formation, do not take place.

Concerning the bulk parameter trends observed for the other cooking processes, reported in **Tables A1**, **A2**, and **A3** (Supporting Information), the most remarkable variation between initial (raw material) and final product values for n_D, d, and total acidity, and H_2O percentage, occurs corresponding to cooking processes C2, C3, and C4. These cooking processes are also characterized by the longest cooking time and the higher temperature values reached at the end of the process. In particular, C2 and C3 cooking processes are conducted in such drastic conditions that the water content at the end decreases to 25−28%.

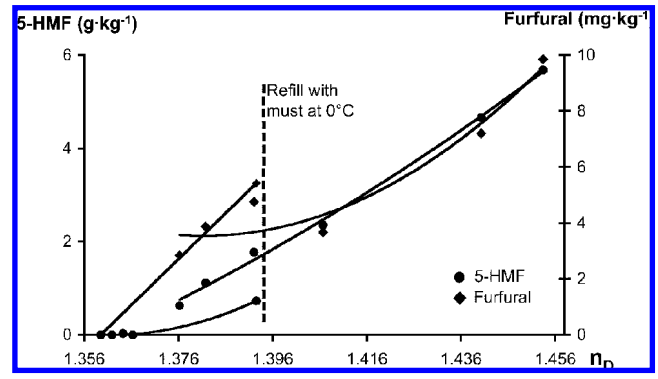

Figure 3. Trend of 5-HMF (-●-) is on the left axis scale and furfural (-◆-) on the right axis scale for cooking process C4 samples depending on their refractive index value (n_D). Concentration values are calculated on dry matter. The dotted line refers to the refill procedure with crude must at 0 °C.

C1 cooking process even though performed by the same producer and procedure with respect to C2, C3, and C4 differs because its cooking process was interrupted during the first night.

Notwithstanding the limited number of cases considered here, it is possible to remark a difference in the total acidity trend between white and red grapes. In fact, when white grape must is cooked an increase in acidity content is observed because of a concentration effect, as shown in **Figure 2** and by the values reported in **Table A1** (Supporting Information). Instead, when red grape must is cooked, both for producers B and C cooking processes, the final products present an acidity value lower than that of the starting products. In particular, in the C5 cooking process a peculiar trend is observed as for total acidity content: two rough falls in acidity values take place (between the 4th and 5th and between the 11th and 12th hours), which interrupt an increasing trend. This could probably be associated with fractionate precipitation and/or complexation phenomena involving organic acids helped by polyphenols species mostly abundant in red grape than in white one.

When compositional data are considered, some evidence can be stressed: (i) the composition of crude musts is very similar concerning sugar content, whereas organic acids content depends on grape variety and origin; (ii) in all crude must samples furfurals are absent, but their production already begins after few hours, especially in a remarkable way for 5-HMF, according to well-known reaction mechanisms such as caramelization and Maillard reactions (*14*); (iii) by taking into account all final products, analogous considerations with respect to those discussed for the bulk parameters hold. The final products of C2, C3, and C4 present the highest values with respect to the other cooking processes C5−C9. In particular, 5-HMF is about 10 times higher, furfural varies from 10 to 50 times, and glucose, fructose, tartaric and malic acids are about 2 times more concentrated. With regard to glucose, fructose, and tartaric and malic acid content, the difference in concentration among cooking processes mostly reflects the different water content: around 30% in C2, C3, and C4 final products and around 60% in C1 and C5−C9 ones.

In order to appreciate the compositional changes independent of the water content variation, concentration values were referred to the dry matter, $C_{DRY} = (C \cdot 100)/\text{water}\%$.

Figure 3 shows the trend of 5-HMF and furfural (concentration values referred to dry matter; data not reported in the **Tables**) for cooking process C4 samples as a function of the refractive index, i.e., n_D values on abscissa. Refractive index

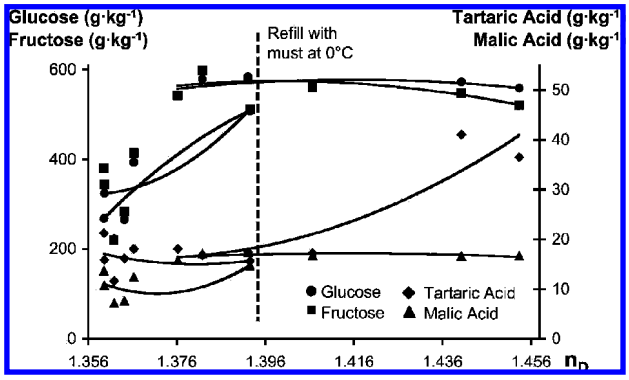

Figure 4. Trend of glucose (-●-) and fructose (-■-) is on the left axis scale and tartaric acid (-◆-) and malic acid (-▲-) on the right axis scale for cooking process C4 samples depending on their refractive index value (n_D). Concentration values are calculated on dry matter. The dotted line refers to the refill procedure with crude must at 0 °C.

also reflects the concentration of the matrix, and it is more suitable to compare trends among different cooking processes.

From **Figure 3**, an increase in furfurals during the cooking process is noticeable also when concentration is referred on dry matter. This is an indirect proof not only that furfurals undergo a concentration effect during heating but also that they are produced by chemical transformations during the entire process. Trends with different slopes can be drawn before (0 to 20th hour interval, corresponding, respectively, to 1.35952 and 1.39257 n_D values) and after (23rd to 43rd hour interval, corresponding, respectively, to 1.37626 and 1.45341 n_D values) the refilling step. In fact, especially for 5-HMF it is possible to observe that its production follows a faster cooking process, increasing with almost an exponential trend, corresponding to lower water contents (second phase of the process) since temperature, which is the other influential variable on furfurals formation, is maintained rather constant during all the processes.

Figure 4 shows the trend of glucose, fructose, and tartaric and malic acids (concentration values referred to dry matter) for cooking process C4 samples as a function of refractive index. By considering the second phase of the process, after refilling, it is possible to observe that malic acid content remains almost constant, in agreement with the absence of specific reactions involving it. Tartaric acid content increases, at variance with what may be expected considering precipitation phenomena. However, it has to be taken into account that even if stored at 0 °C must solutions are not stable. Because it was observed by visual inspection of samples that showed varying amounts of precipitate and suspended particulate, this trend may depend also on the time a given sample has been analyzed besides the time position it occupies in the cooking process when it was collected. It is possible to arrive at the same conclusion by a deeper examination of the total acidity and tartaric and malic acid data. In fact, although the sum of the fixed acids ought to be coherent with the titrated acidity (expressed in the same scale unit) in the case of the less cooked products, such as the samples C5 from 0 to 4 h, it is possible to note a discrepancy in the experimental data. This fact can be interpreted by the formation

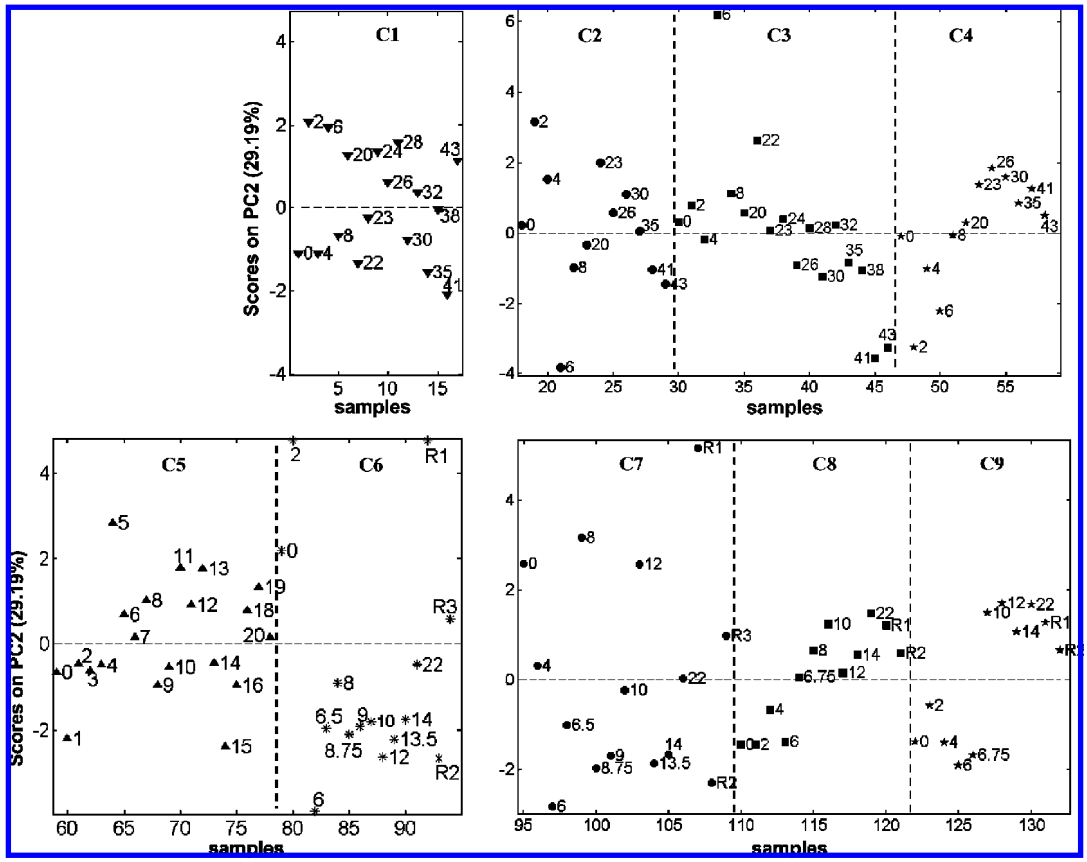

Figure 5. PCA results: scores on PC1 vs samples. Samples are arranged from 1 to 132 according to cooking process order (from C1 until C9). Cooking processes are labeled by the letter C followed by the number of identification (see Tables A1−A3, Supporting Information), whereas samples are labeled with a number indicating the time of heating (expressed in hours). Refill samples used in C6−C9 cooking processes are labeled with R1, R2, and R3 (respectively first, second, and third refilling procedure). For sake of clarity, the original figure was split into 4 regions according to sample score values, then cooking processes with similar values were considered together, by using sketched lines as separators: in the upper panels, there are results relative to C1−C4, and in the lower panel, there are results relative to C5−C9.

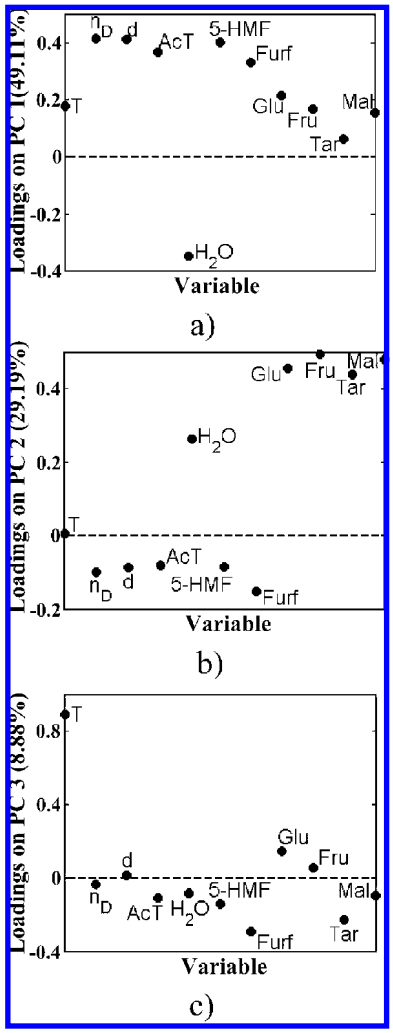

Figure 6. PCA results: (**a**) loadings on PC1, (**b**) PC2, and (**c**) PC3 vs variables. Eleven variables were arranged in the same order in Tables A1−A3 (Supporting Information).

of acetic acid in the sample that is evaluated by the titration but not detected with the GC method (*23*). Concerning the relevance of the fermentation phenomena, we might reasonably think that this is strictly related to the time between the sampling and the chemical analysis.

Moreover, complexation/precipitation equilibria may influence each other in a complex way, given the nature of the studied matrix. As far as reducing sugars are concerned, particularly fructose, a decreasing trend is observed; this can reasonably be related to the furfural formation reaction that involves the consumption of the sugar substrate. Moreover, the major reactivity of fructose to give degradation reactions with respect to glucose is also confirmed.

PCA Analysis. PCA analysis was applied to all the available data with the aim of (i) studying the role of the different technological parameters/cooking strategies adopted by the producers in the must transformation occurring during the heating process; (ii) comparing the trends showed by the different cooking processes, depicting both their peculiar characteristics and the common trends.

For these purposes, the chemical variables (i.e., furfurals, sugars, and organic acids) used in PCA analysis were referred to dry matter, as previously described. Before arranging the data set in a suitable matrix for the chemometrics analysis, an outliers investigation was performed by using a previous explorative PCA analysis. On the basis of these results, some experimental data were considered as outliers and removed from the data set (see **Tables A1−A3**, Supporting Information).

Looking at the samples listed in **Tables A1−A3** (Supporting Information; 122 samples), it emerges that some samples, such as crude must and must samples used for refilling, are common to more cooking processes, i.e., those of the same producer. In the data set subjected to PCA, these common samples are repeated because of the type of scaling adopted. In fact, each subset corresponding to one cooking process has been mean centered separately, resulting in 9 mean centered data matrices of dimensionality, R-rows (number of samples of a given cooking process) × 11 variables (T, n_D, d, total acidity, H_2O%, 5-HMF, furfural, glucose, fructose, and tartaric and malic acid concentrations referred to dry matter), and then the 9 data matrices have been assembled into a unique one (132 rows × 11 columns) and autoscaled.

In this way, the trend inside each cooking process can be highlighted since each sampled point is anchored to the average of its own cooking process, while maintaining the differences among the various cooking processes.

Figure 5 shows the scores plot for the first component that explains 49.11% of cumulative variance. For the sake of clarity, the **Figure** is split into four subsections as described in detail in the caption.

PC1 score values distinguish cooking processes into two groups according to the range of variation passing from crude must to the final product, i.e., cooked reduced must: C2, C3, and C4 cooking processes (top panel) show an interval of about 15 units in the PC1 score range, while the score range for the other cooking processes covers almost a 4 unit interval. This difference reflects the heating time, in fact C2−C4 products were cooked over 40 h and C5−C9 products about 20 h. Because of the accidental stopping of heating, C1 belongs to the second group, even if total duration in time is 43 h. Thus, PC1 orders samples according to the number of hours of heating, assigning negative values to crude musts and intermediate cooked musts, and positive values to rather cooked musts and final products. The trends observed for C6−C9 samples are less regular. This is probably due to the refilling operation performed 2−3 times by using partially cooked musts. However, the larger interval in PC1 score values for C2−C4 cooking processes is an index of a greater variability inside these cooking processes, a consequence of a greater variation in sample composition during the heating process; the location of samples also shows how the points of refilling come close to the lesser cooked samples.

Moreover, the must samples used for the first refills in cooking processes performed by producer C (i.e., R1, in cooking process C6 and C7; R1 and R2 in cooking process C8 and C9) present a particular location, i.e., shows PC1 score values higher than the samples taken just before refilling. This could be explained considering the different capacities and technologies of pans used in the must cooking process with respect to the small pans where must to be used for refilling was heated (see the Sampling section). The bigger pan equipped by a stirrer device obviously gives the best (exposed-to-air surface/total bulk volume) ratio, which aids homogenization of the product and as consequence a more controlled processes, whereas the smaller pan without an agitation system leads to most marked reactions and variations. Moreover, refill samples were cooked at a higher temperature (≥89 °C instead of ≤80 °C). By analyzing the PC1 loadings (**Figure 6a**), it is possible to note that all variables, except the water content (H_2O%), present positive values. Thus,

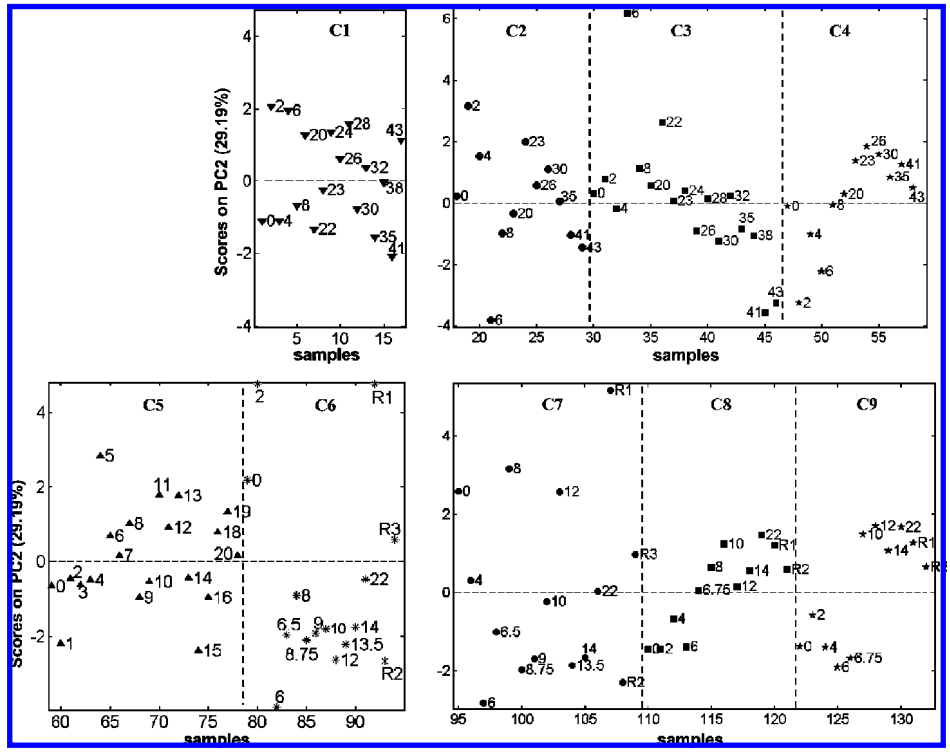

Figure 7. PCA results: scores on PC2 vs samples. Cooking processes are labeled by the letter C followed by the number of identification (see Tables A1−A3, Supporting Information), whereas samples are labeled with a number indicating the time of heating (expressed in hours). Refill samples used in C6−C9 cooking processes are labeled with R1, R2, and R3 (respectively, first, second, and third refilling procedure). For sake of clarity, the original figure was split into 4 regions according to sample score values, then cooking processes with similar values were considered together by using sketched lines as separators: in the upper panels, there are results relative to C1−C4, and in the lower panels, there are results relative to C5−C9.

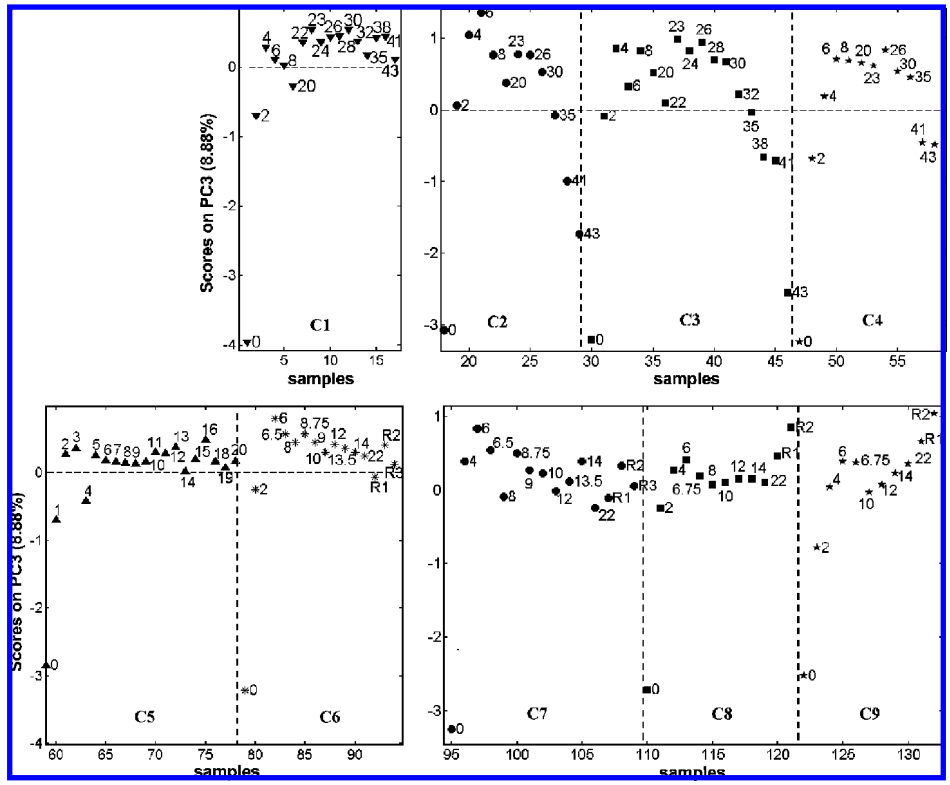

Figure 8. PCA results: scores on PC3 vs samples. Cooking processes are labeled by the letter C followed by the number of identification (see Tables A1−A3, Supporting Information), whereas samples are labeled with a number indicating the time of heating (expressed in hours). Refill samples used in C6−C9 cooking processes are labeled with R1, R2, and R3 (respectively, first, second, and third refilling procedure). For sake of clarity, the original figure was split into 4 regions according to sample score values, then cooking processes with similar values were considered together by using sketched lines as separators: in the upper panels there are results relative to C1−C4, and in the lower panels, there are results relative to C5−C9.

by comparing with the PC1 scores plot, it is possible to associate lower values in water content with higher values in n_D, d, total acidity, and chemical component content to most cooked samples and vice versa for crude and intermediate cooked musts. Moreover, it is worth noticing that the chemical species characterized by greater variation, during the entire cooking process, are 5-HMF and furfural.

PC2 and PC3 score plots are reported, respectively, in **Figures 7** and **8**. PC2 (29.19% explained variance) only in some cases allows to differentiate samples on the basis of cooking time and mostly highlights the peculiar behavior of some of the samples. The PC2 loadings plot (**Figure 6b**) shows two groups of variables: at high positive values variables that tend to have oscillating values, such as sugars and tartaric and malic acids, and at most negative values variables that tend to increase during heating, such as furfurals. Total acidity is on this group because its values are not corrected for the contribution of water. It is also interesting to note the opposite position of water content with respect to furfurals.

Finally, PC3 (accounting for 8.88% of total variance) is mostly influenced by temperature (high positive loading value); consequently, crude must samples, which are characterized by very low T values, are positioned far (most negative scores values) from all of the other samples, which result close together and to the origin (**Figure 6c**). The most cooked must samples and final products of C2−C4 cooking processes show intermediate negative score values because of significant amount of furfurals (most negative loading values).

The obtained results have evidenced strong variations, both in the chemical composition and in physical properties of the matrix, during the heating process. These also depend on the technological apparatus and on the process strategy. The presence of furfurals in cooked reduced must, on the one hand, confers peculiar positive organoleptic characteristics to the ABTM product obtained by it, but might represent a negative aspect for the safety of the product. As for the potentially noxious and tumorigenic activities, together with cytotoxis and genotoxic activities, they also render the cooked must an unfavorable substrate for bacteria and micro-organisms involved in alcoholic fermentation and acetic bio-oxidation reactions that have to occur to mature ABTM. Therefore, the identification of the optimal conditions in must heating processes in order to control the content of furfurals inside acceptable limits represents an extremely important issue. Notwithstanding, the definition of the set of conditions that cause a perfectly controlled process is still premature. Relevant evidence emerged that drastic conditions, such as the combination of a prolonged heating time together with reaching temperature values higher than 90 °C and water content lower than 40%, strongly favor furfural production, especially 5-HMF. In fact, the sugar dehydration reaction occurs in a more pronounced and, in worse cases, uncontrolled way, if the loss of water takes place in the presence of high sugar content and acid conditions.

Therefore, as far as the definition of new operative conditions for the production of reduced cooked musts is concerned, on the basis of these experimental data, it is possible to hypothesize that high temperature, close to or higher than 90 °C, could be used during the beginning of the process when water content is still close to 80%. Then, as the water content starts decreasing, the temperature must be set to a lower value (70−80 °C) until the final sugar concentration (350−400 g/kg) is obtained. It is worth noticing that the sugar concentration of 350−400 g/kg represents the suitable value for the starting raw material for ABTM production.

This work represents a first attempt to give to producers useful knowledge in order to obtain products with complete respect not only to the traditional protocol but also to quality and safety assurances. In this context, a new project has started in order to verify the above-mentioned operative conditions for the production of reduced cooked musts.

Supporting Information Available: The experimental data, evaluated on food samples investigated in the present research. In particular, Table A1 reports temperature (T/°C), refractive index (n_D), density (d/g·cm^{-3}), total acidity ($g_{acetic\ acid}$ in 100 g_{sample}), water content (H_2O/%), 5-HMF, furfural, glucose, fructose, tartaric acid, and malic acid (mg·kg^{-1}) values for the cooking processes C1−C4 performed simultaneously by Producer A. Table A2 reports the same parameters for the cooking process C5 from Producer B, and finally, Table A3 reports the same parameters for the cooking processes C6−C9 performed by Producer C. This material is available free of charge via the Internet at http://pubs.acs.org.

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